IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Weiss *et al.*
Serial No.	:	08/486,313
Filed	:	June 7, 1995
For	:	MULTIPOTENT NEURAL STEM CELL COMPOSITIONS
Examiner	:	A-M. Baker
Group Art Unit	:	1632

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132

I, E. EDWARD BAETGE, hereby declare and state as follows:

1. I received my B.S., and Ph.D. degrees from the University of San Diego & Cornell University in 1978 and 1983, respectively. I am a named inventor. I have been working in the field of cell and molecular neurobiology since 1978, and working with neural stem cells since 1991.

2. I am currently employed as Chief Scientific Officer at Modex Therapeutiques SA (Lausanne, Switzerland) in the field of cell and molecular Biology. Prior to this, I worked at CytoTherapeutics, Inc. (now StemCells, Inc.), the company that is the exclusive licensee of this patent application. At CytoTherapeutics, I was in charge of all scientific research. I am a named inventor on this patent application and am intimately familiar with the neural stem cell cultures and methods of using them that this application describes (such as the pending claims which are directed to methods for transplanting neural stem progeny into a host).

3. I understand that the Examiner has rejected the pending claims under 35 U.S.C. §112 contending that "[t]he claims are not enabled because the transplantation of multipotent neural stem cell progeny into a host has not been demonstrated to provide any therapeutic benefit to the host."

4. I make this declaration to rebut the Examiner's rejection, with which I do not agree. In view of the express statements in the specification regarding transplantation of neural stem cell cultures and the voluminous experimental evidence that has been accumulated, in my opinion, the ordinarily skilled artisan would be able to routinely transplant the described neural stem cell cultures without

undue experimentation at the filing date of this application. I am also of the opinion that although the claimed methods do not state or require any therapeutic benefit, that transplantation of neural stems into a host has been clearly demonstrated to confer a therapeutic benefit, and that the ordinarily skilled artisan would believe that such transplantation would provide a therapeutic benefit to the host.

5. Prior to this invention, the operating dogma in neurobiology was that the brain was relatively quiescent, and that there was no "stem cell" that could be proliferated and then differentiated to form the three major cell types in the central nervous system (*i.e.*, neurons, astrocytes and oligodendrocytes). The neural stem cells described for the first time in this invention, and the ability for the art (provided by the inventors here) to obtain proliferating cultures of those cells, has been widely hailed as a landmark in neurobiology. In fact, over 500 published references cite one of the early publications that describe this invention (Reynolds and Weiss, Science 255:1707-10 (1992)). A number of these publications demonstrate that a therapeutic benefit is conferred when neural stem cells are transplanted into a host. I will discuss these in detail below.

6. As a general matter, it is my view, based on my knowledge of the field and based on the voluminous citations to the inventors' work, that researchers of ordinary skill in the relevant arts clearly recognized the importance of the Applicants' invention and relied upon it in their subsequent work. In my opinion, the Applicants' invention discloses paradigm-shifting technology and is a vitally important finding in the field of neurobiology. I am of the firm view that the claimed methods for transplanting neural stem cells are enabled, and provide my detailed thoughts below.

7. I believe that the neural stem cell cultures of this invention are particularly suited for transplantation, since, until this invention, cultures of proliferating neural cells have not been available to the art. Further, in the claimed transplantation methods, the tissue source is well-defined, reproducible, and is not derived from an oncogene-immortalized cell line (thus being non-tumorigenic). *See,* specification, pg. 11, lines 15-20. In fact, I concluded some years ago that such neural stem cell cultures are "ideal" for nervous system transplantation. *See, Baetge et al.,* 695 Ann N.Y. Acad. Sci., pp. 285-291 (1993). Multiple other workers in the field have reached the same conclusion.

8. I understand that the specification contains explicit guidance about how to transplant CNS neural stem cells (*see,* specification, pg. 36, line 10, to pg. 42, line 13; pg. 68, line 16, to pg. 69, line 18; pg. 78, line 17, to pg. 71, line 6; pg. 96, line 12, to pg. 97, line 28), and that the specification provides working examples of neural stem cell transplantation in various disease models, including, *e.g.*, Huntington's disease, Parkinson's disease, and cardiac arrest. *See, e.g.*, specification, pp. 96-101. In addition, the specification teaches and discloses the types of diseases to which the invention is directed. (*See* pg. 40, lines 9-18). The specification also provides exemplary teaching of where to transplant the cells of the claimed invention. (*See, e.g.*, pg. 38, lines 17-30). Further, the specification teaches and discloses how to monitor the transplanted cells. (*See* pg. 39, lines 16-31). Additionally, the specification teaches and discloses how to get the transplanted cells to proliferate *in vivo*. (*See* pg. 52, line 14 through page 47, line 26). In addition, I believe that the ordinarily skilled artisan would know how to actually carry out the step of transplanting the neural stem cell cultures of this invention according to the claimed methods given that the art is replete with examples of transplantation of various other tissues or cells into various parts of the brain (witness, e.g., transplantation of porcine neural cells and fetal human cells). For this reason, in my view it cannot be disputed that the ordinarily skilled artisan, with the specification in hand, would be able to transplant neural stem cell cultures into a host; that is, the ordinarily skilled artisan would know how to use the invention as claimed. I do not believe that the Examiner disputes this; rather I believe that the Examiner is asking for additional proof that the ordinarily skilled artisan would believe that making such a transplantation would confer a therapeutic benefit. I have provided that proof below.

9. I understand that the specification provides forty-five (45) *in vitro* and *in vivo* examples relating to generation and use of neural stem cell cultures. Among these examples are detailed various standard, well-accepted animal models of various human diseases, including, *e.g.*, animal models for Parkinson's disease and Huntington's disease. Applicants also disclosed treatment of neurodegenerative disease using progeny of human neural stem cells proliferated *in vitro*; remyelination in myelin deficient rats using neural stem cell progeny proliferated *in vitro*; remyelination in human neuromyelitis optica; and remyelination human Pelizaeus-Merzbacher disease. (*See* Specification, Examples 14-17).

10. I have read Dr. Hammang's December 2000 Declaration and I agree with his views expressed therein. In particular, Dr. Hammang gave two reasons why the ordinarily skilled artisan

would reasonably expect that the claimed transplantation methods would provide a therapeutic benefit; (1) that the transplanted neural stem cell cultures secrete cellular products which are capable of providing a therapeutic benefit to the host, and (2) that the neural stem cell cultures exhibit tissue-specific differentiation upon transplantation. I agree with Dr. Hammang. In my view, either of these facts would inescapably lead the ordinarily skilled artisan to conclude that transplantation of such neural stem cell cultures would have a reasonable expectation of success in providing a therapeutic benefit to the host.

A. Transplantation of Neural Stem Cell Progeny According to the Claimed Methods For Delivery of Cellular Products Provides a Therapeutic Benefit

11. Applicants' neural stem cell cultures have been shown to be a useful tool for delivery of secreted cellular products which provide a therapeutic benefit when transplanted into the host. I draw the Examiner's attention to three publications that demonstrate that transplantation of cultures of neural stem cells that have been genetically modified to secrete nerve growth factor ("NGF"), according to the claimed methods, provides a therapeutic benefit.

12. Andsberg et al., "Amelioration Of Ischaemia-Induced Neuronal Death In The Rat Striatum By NGF-Secreting Neural Stem Cells", Europ. J. Neuroscience, 10, pp. 2026-2036 (1998) (copy attached as Ex. 1) reports that transplantation of NGF-secreting neural stem cell cultures into the adult rat striatum following middle cerebral artery occlusion ameliorated the death of striatal projection neurons that would have otherwise died due to the ischaemic insult. This clearly demonstrates a therapeutic benefit of the claimed methods.

13. Carpenter et al, "Generation and Transplantation of EGF-responsive Neural Stem Cells Derived From GFAP-hNGF Transgenic Mice", Exp. Neurology, 148, pp. 187-204 (1997) (copy attached as Ex. 2) reports that transplantation of NGF-secreting neural stem cell cultures into the adult rat striatum produced dense sprouting of p75 neurotrophin receptor-positive fibers emanating from the underlying basal forebrain – a significant morphological change compared to controls, which I believe would be considered a therapeutic benefit in hosts where such neuronal regeneration and sprouting were desired. The authors conclude that "[t]he use of neural stem cells for transplantation into the CNS offers a number of advantages over transplantation of primary tissue or other cell lines." *See* p. 202. This to me also clearly demonstrates a therapeutic benefit of the claimed methods.

14. Kordower et al., "Grafts of EGF-responsive Neural Stem Cells Derived From GFAP-hNGF Transgenic Mice: Trophic and Tropic Effects in a Rodent Model of Huntington's Disease", J. Comp. Neurol., 387, pp. 96-113 (1997) (copy attached as Ex. 3) reports that intrastriatal transplantation of NGF-secreting neural stem cell cultures into an adult rat model of Huntington's disease (the well accepted and widely used quinolinic acid lesion model) resulted in sparing of striatal neurons immunoreactive for glutamic acid decarboxylase, choline acetyltransferase, and neurons histochemically positive for nicotinamide adenosine diphosphate. In addition the NGF-secreting transplants produced robust sprouting of cholinergic fibers from subjacent basal forebrain neurons. The authors conclude that "[t]hese data indicate that cellular delivery of hNGF by genetic modification of stem cells can prevent the degeneration of vulnerable striatal neural populations, including those destined to die in a rodent model of HD and supports the emerging concept that this technology may be a valuable therapeutic strategy for patients suffering from this disease." *See* p. 96. In my view, the authors clearly demonstrated a therapeutic benefit and clearly expressed their view that the results in the model are predictive of the human condition. For this reason, these data also clearly demonstrates a therapeutic benefit of the claimed methods.

B. Transplantation of Neural Stem Cell Progeny According to the Claimed Methods For Tissue-Specific Differentiation Provides a Therapeutic Benefit

15. Applicants' neural stem cell cultures have been shown to be useful tool for tissue-specific differentiation to provide a therapeutic benefit when transplanted into the host. I draw the Examiner's attention to several new publications that demonstrate that transplantation of cultures of neural stem cells provide such a therapeutic benefit.

16. Qu et al., "Human Neural Stem Cells Improve Cognitive Function of Aged Brain", Ageing, 12, pp. 1127-1132 (2001) (copy attached as Ex. 4) report that when human neural stem cells were transplanted into aged rats (about 24 months old), according to the claimed methods, the cells not only survived, but also retained their multipotency and migratory ability. The results show that the human neural stem cells not only successfully differentiated into neurons and astrocytes, but importantly "both neurons and astrocytes migrated into the cortex and hippocampus in a well-defined and organized pattern in the brain." *See* p. 1132. Finally, the results demonstrate

significantly improved cognitive function (in the standard and well accepted Morris water maze model). In my view this clearly demonstrates a therapeutic benefit of the claimed methods.

17. Akiyama et al., "Transplantation of Clonal Neural Precursor Cell Derived From Adult Human Brain Establishes Functional Peripheral Myelin in the Rat Spinal Cord", Exp. Neurol., 167, pp. 27-39 (2001) (copy attached as Ex. 5) reports that human neurosphere cultures (*i.e.*, an expressly disclosed embodiment of the neural stem cell cultures of this invention) when transplanted (according to the claimed methods) into a demyelinated adult rat spinal cord produced extensive remyelination with a peripheral pattern similar to Schwann cell myelination characterized by large cytoplasmic and nuclear regions, a basement membrane, and P0 immunoreactivity. Importantly, "the remyelinated axons conducted impulses at near normal conduction velocities". *See* p. 27. In my view this clearly demonstrates a therapeutic benefit of the claimed methods.

18. Kurimoto et al., "Transplantation of Adult Rat Hippocampus-Derived Neural Stem Cells into Retina Injured By Transient Ischemia", Neuroscience Letters, 306, pp. 57-60 (2001) (copy attached as Ex. 6) reports transplantation of rat neural stem cell cultures into the eyes of adult rats that underwent ischemia-reperfusion injury. The *in vivo* retinal ischemia-reperfusion model is a standard (and well accepted) experimental model that has been used to investigate the damage to the retina induced by transient ischemia. The authors report that in the eyes with the ischemia insult, the intravitreally injected neural stem cells invaded the retinal ganglion cell layer within a week of the transplantation and were identified in the retinal inner nuclear layer two weeks after the transplantation. At four weeks the donor cells were integrated into the host retina and expressed Map2ab, which indicated that the cells had differentiated into mature neurons. By comparison, in the control, none of the transplanted cells migrated to the retina. The authors conclude that "neuronal stem cells are good candidates to reconstruct the neural circuitry of ischemic injured retina, and show the potentiality of therapeutic transplantation using neuronal stem cells on retinal impairments that are generally regarded as incurable." *See* p. 59. I conclude from this that, both the authors (and myself) believe that this clearly demonstrates a therapeutic benefit of the claimed methods.

19. Likewise, Nishida et al., "Incorporation and Differentiation of Hippocampus-Derived Neural Stem Cells Transplanted in Injured Adult Rat Retina", Investigative Ophthalmology & Visual Science, 41, pp. 4268-4274 (Dec 2000) (copy attached as Ex. 7) report that transplantation of neural

stem cells into mechanically injured adult retina results in incorporation and subsequent differentiation of the grafted stem cells into neuronal and glial lineages. Importantly, the authors conclude that "[n]eural stem cells are expected to be useful clinically for replacing damaged neurons or for ex vivo gene therapy." *See*, p. 4271. This statement confirms my statements throughout this declaration, and I believe is reflective of the general opinion of those of ordinary skill in the art.

20. I also refer the Examiner to Milward *et al.*, 50 J. Neurosci. Res. 862-871 (1997) ("*Milward*") (Ex. 8). *Milward* successfully transplanted canine CNS neural stem cells both into rat and into a shaking (*sh*) pup myelin mutant dog (a model of human myelin diseases). In *Milward*, canine neural stem cell cultures were transplanted into the myelin-deficient (*md*) rat spinal cord, resulting in the production of myelin by graft-derived cells (*see, Milward,* pg. 868, col. 1, 2nd para.), demonstrating that transplanted CNS neural stem cells can differentiate in the recipient to form myelin-producing oligodendrocytes and therapeutically provide myelin to recipients. The *Milward* shaking pup canine model is a particularly harsh model of dys-myelination. The pups are born with little to no myelin sheath. The *Milward* data demonstrate that that the present invention can be used to provide myelination in an almost myelin-free *in vivo* environment. In addition, as *Milward* reports, the grafted cells had integrated normally into the adult *sh* pup cytoarchitecture. *See*, p. 867, right column. This is powerful support that transplantation of such neural stem cell cultures would be useful in providing a therapeutic benefit to the host. This is particularly true in "real-life" disease situations where de-myelination is a relatively slow process occurring not globally but in patches.

The Akiyama paper discussed above confirms that the remyelination effected by transplantation of neural stem cells (as demonstrated in *Milward*), in fact, is functional and therapeutic (as it restores near normal conduction velocities to the remyelinated axons).

I understand that the Examiner has indicated that "the formation of myelin, as described by Milward *et al.*, did not result in producing a therapeutic effect in the animal. Thus, Milward *et al.* does not demonstrate application of the claimed method to produce a therapeutic effect." (Office Action, pages 4-5). I disagree with the Examiner's characterization of *Milward*, and, it is my opinion that the Akiyama paper discussed herein confirms my earlier conclusions regarding Milward. In my view, the ordinarily skilled artisan would expect that replacement of myelin on demyelinated axons would confer a therapeutic benefit, since the "replaced" myelin provides the missing insulation that is necessary to support impulse conduction. The Examiner does not explain

why she believes that *Milward* does not produce a therapeutic effect, and I believe that Akiyama directly supports that a therapeutic benefit is conferred.

21. I also refer the Examiner to Zhang *et al.*, 96 Proc. Natl. Acad. Sci. USA 4089-94 (1999) ("*Zhang*") (Ex. 9). *Zhang* reports similar results, in which neural stem cell cultures were generated from both juvenile and adult rats and used to produce myelin-forming cells, and when transplanted into *md* rats, those cells produced "robust myelination". *See, Zhang*, pp. 4093-94. For the reasons discussed above, this too, in my view, would lead the ordinarily skilled artisan to conclude that transplantation of such neural stem cell cultures would be useful in providing a therapeutic benefit to the host.

I understand that the Examiner has stated that "this 'robust myelination' in fact did not produce a therapeutic effect in the host." (Office Action, page 5) However, the Examiner has not provided any evidence for this assertion. Moreover, I disagree with the Examiner's contention. As noted above, the ordinarily skilled artisan would expect that replacement of myelin to demyelinated axons would confer a therapeutic benefit. I believe that Akiyama directly supports that a therapeutic benefit is conferred.

22. Likewise, I also refer the Examiner to Brüstle *et al.*, 16 Nature Biotechnol., pp. 1040-1044 (1998) ("*Brüstle*") (Ex. 10). *Brüstle* describes the implantation of fetal human CNS progenitor cells into mice that "acquire an oligodendroglial phenotype and participate in the myelination of host axons".

The Examiner has stated that, because *Brüstle's* experiments were conducted in healthy animals, no therapeutic effect was demonstrated because the "function of the cells upon transplantation is not sufficient to support enablement because there is not sufficient guidance for using the transplantation method therapeutically in diseased animals." (Office Action, page 5).

I disagree. *Brüstle* did demonstrate that the transplanted cells functioned as expected after transplantation – and participated in the myelination of host axons. I believe that demonstration of ability to myelinate axons (as *Brüstle's* experiments showed) would lead one of ordinary skill in this art to reasonably expect that these cells would provide a therapeutic benefit in the appropriate disease environment. Additionally, in view of Akiyama, I do not believe the Examiner can maintain the position that the claimed methods do not produce a therapeutic benefit in demyelinating/dismyelinating disorders.

23. Others have also demonstrated that these neural stem cell cultures, when transplanted, are, in fact, capable of replacing the critical functions of lost or deficient neural populations. *See, e.g.*, Flax *et al.*, 16 Nature Biotechnol., pp. 1033-1039 (1998) ("*Flax*")(Ex. 11). *Flax* showed that transplantation of CNS neural stem cells provides a therapeutic benefit in the meander tail (*mea*) mouse, a well-known and well accepted mouse mutant model characterized by a cell-autonomous failure of granule neurons to develop or survive in the cerebellum, especially the anterior lobe. *Flax* transplanted human CNS neural stem cells into newborn *mea* cerebella and confirmed that the human neural stem cells provided "replacement neurons" with the "definitive size, morphology, and location of cerebellar granule neurons (Fig. 6E-G)" (*Flax*, pg. 1037, col. 2,2nd para).

Thus, in my view, *Flax* showed that transplanted CNS neural stem cells are able to differentiate in the recipient to form granule neurons and to therapeutically provide replacement neurons to recipients (such as these *mea* mice). In my view, this ability to replace damaged or missing neurons is unequivocal evidence of a therapeutic benefit upon transplantation of neural stem cell cultures, as claimed here. Moreover, my view has also been supported independently by other workers in the field who reviewed this *Flax*, Nature Biotech paper. *See* Zigova & Sanberg, 16 Nature Biotechnol., pp. 1007-1008 (1998) ("*Zigova*") (Ex. 12) which states that the *Flax* data "provides strong evidence that the NSCs [neural stem cells] are able to perform *in vitro* and *in vivo* all the critical functions previously described for their rodent counterparts" (*Zigova*, pg. 1007, middle column).

I understand that the Examiner has indicated that the "'replacement neurons' in fact did not produce a therapeutic effect. Thus, it is unclear how the claimed method can be used for therapy, since no teachings are provided to allow the skilled artisan to produce 'replacement neurons' for therapy." (Office Action, page 6). I disagree with this position. *Flax* transplanted NSCs into a murine model characterized by a failure of granule neurons to develop or survive in the cerebellum. The results of these experiments with this model demonstrate that the NSCs differentiated into neurons of the appropriate size, morphology, and location. *Flax* concluded that "engrafted NSCs of human origin appear sufficiently plastic to respond appropriately to varying local cues for lineage determination."

Thus, in this animal model that is lacking a certain type of neuron, *Flax* demonstrated that transplantation of neural stem cell cultures replaced the missing neurons. In my opinion, these results are clear evidence of the therapeutic effect of the claimed invention because the results demonstrate that transplantation of neural stem cells according to the claimed methods results in tissue-specific differentiation.

24. I also referred to Fricker *et al.*, 19 J. Neurosci., pp. 5990-6005 (1999) ("*Fricker*") (Ex. 13). *Fricker* showed that when CNS neural stem cells were transplanted into neurogenic regions in the adult rat brain, the subventricular zone, and hippocampus, the *in vitro* propagated cells migrated specifically along the routes normally taken by endogenous neuronal precursors: along the rostral migratory stream to the olfactory bulb and within the subgranular zone in the dentate gyrus. The *in vitro* propagated cells exhibited site-specific neuronal differentiation in the granular and periglomerular layers of the bulb and in the dentate granular cell layer. Additionally, the CNS neural stem cells also exhibited substantial migration within the non-neurogenic region, the striatum, and showed differentiation into both neuronal and glial phenotypes. Thus, in my view, *Fricker* confirmed the ability of the human neural stem cells to respond *in vivo* to guidance cues and signals that can direct their differentiation along multiple phenotypic pathways.

The Examiner noted that the ability of human NSCs to respond *in vivo* to guidance cues and signals that can direct their differentiation "has not as yet been exploited to develop methodology to produce a therapeutic effect." (Office Action, page 6). Again, I do not agree with this characterization. In my view, one skilled in the art would recognize the importance of *Fricker*'s discovery that the NSC cultures are able to respond *in vivo* to surrounding signals and cues in the treatment of a variety of CNS diseases and disorders. Thus, it is my opinion that the work reported by *Fricker* could be used by those skilled in the art to achieve a therapeutic benefit in a patient being treated.

25. I also draw the Examiner's attention to Aboody et al., 97 Proc. Natl. Acad. Sci. USA, pp. 12846-51 (November 2000) ("*Aboody*") (Ex. 14). *Aboody* expressly states (*see, e.g.*, throughout the paper, and particularly Abstract and p. 12851) that neural stem cell cultures provide a transplantation "platform" since upon transplantation those cells can both continue to express a foreign gene and migrate in a site specific fashion in host tissue for "dissemination of therapeutic genes".

In response, the Examiner has states that "Aboody et al., does not teach the steps required to transplant NSCs in a manner such that a therapeutic effect is produced." (Office Action, pages 6-7). To the contrary, it is my belief that *Aboody*'s characterization of the neural stem cell cultures as a transplantation "platform" is sufficient evidence of a therapeutic benefit -- specifically, those skilled in the art would be able to use these cells in the claimed methods to achieve such a therapeutic benefit without undue experimentation.

26. Finally, I draw the Examiner's attention to several additional publications that, in my view, clearly demonstrate that the art is of the view that the claimed transplantation methods would provide a therapeutic benefit. In particular, *see e.g.* Ourednik et al., Novartis Foundation Symposium 231, Pub. John Wiley & Sons, Ltd. (2000) (Ex. 15), which is titled "Neural Stem Cells Are Uniquely Suited For Cell Replacement and Gene Therapy in the CNS". This title alone captures the sentiments expressed throughout my declaration. *See* also Vescovi et al., "Isolation and Intracerebral Grafting of Nontransformed Multipotential Embryonic Human CNS Stem

Cells", J. Neurotrauma, 16, pp. 689-693, p. 689 (1999) (Ex. 17), which states "the use of human embryonic CNS stem cells should provide a reliable solution to some of the major problems that pertain to this field ...".

27. For all the foregoing reasons, I believe that the Examiner should withdraw the rejection and allow the pending claims.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, Title 18, United States Code, and that willful false statements may jeopardize the validity of this application and any patent issuing therefrom.

E. Edward Baetge

Signed at Lausanne, Switzerland
this 30 day of July, 2001

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Amelioration of ischaemia-induced neuronal death in the rat striatum by NGF-secreting neural stem cells

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Keywords: ischaemia, *ex vivo* gene transfer, gene therapy, nerve growth factor, stroke

Abstract

The objective of the present study was to explore whether grafted immortalized neural stem cells, genetically modified to secrete nerve growth factor (NGF), can ameliorate neuronal death in the adult rat striatum following transient middle cerebral artery occlusion (MCAO). One week after cell implantation in the striatum, animals were subjected to 30 min of MCAO. Striatal damage was evaluated at the cellular level after 48 h of recirculation using immunocytochemical and stereological techniques. The ischaemic insult caused an extensive degeneration of projection neurons, immunoreactive for dopamine- and adenosine 3′: 5′-monophosphate-regulated phosphoprotein with a molecular weight of 32 kilodaltons (DARPP-32). ^{3}H-Thymidine autoradiography demonstrated surviving grafted cells in the lesioned striatum in all transplanted rats. The loss of striatal projection neurons was significantly reduced (by an average of 45%) in animals with NGF-secreting grafts, whereas control cells, not producing NGF, had no effect. The neuroprotective action of NGF-secreting grafts was also observed when the total number of striatal neurons immunopositive for the neuronal marker NeuN was quantified, as well as in cresyl violet-stained sections. The present findings indicate that administration of NGF by *ex vivo* gene transfer and grafting of neural stem cells can ameliorate death of striatal projection neurons caused by transient focal ischaemia.

Introduction

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) belong to a family of highly bioactive peptides, called neurotrophins, with a broad range of effects on neurons, including protective actions (Lindvall *et al.*, 1994; Lewin & Barde, 1996, for review). The expression of the neurotrophins and their receptors is regulated by a variety of acute insults to the brain, including epileptic seizures, hypoglycaemic coma, cerebral ischaemia and traumatic injury (Gall & Isackson, 1989; Ballarín *et al.*, 1991; Ernfors *et al.*, 1991; Lindvall *et al.*, 1992; Merlio *et al.*, 1993; Mudó *et al.*, 1993). The increased synthesis of NGF and BDNF triggered by these conditions has been interpreted as an intrinsic neuroprotective response of the damaged brain (Lindvall *et al.*, 1994; Koistinaho & Hökfelt, 1997). This has raised the possibility that administration of one or more of the neurotrophins might be a useful strategy to protect injured neurons from dying in the event of an acute neurodegenerative disorder. In support of this idea, exogeneously supplied NGF has been reported to counteract death of hippocampal neurons exposed to hypoglycaemia *in vitro* (Cheng & Mattson, 1991) and of CA1 neurons following transient global forebrain ischaemia in rats *in vivo* (Shigeno *et al.*, 1991; Pechan *et al.*, 1995), although this was not observed by others (Beck *et al.*, 1992).

Biological delivery of neurotrophic factors is an effective approach for administering these peptides locally into the mammalian brain. Their poor diffusion properties, short half-life in the brain interstitium, and the physiological limit that the blood brain barrier represents for the systemic administration of neurotrophic factors, have previously made it necessary to use intraventricular or intracerebral injections or infusions. Such procedures often cause traumatic, chronic brain injury and, in addition, are of limited duration. Recently developed techniques for gene transfer, especially those based on *ex vivo* gene transfer of transgenic neurotrophic proteins to different types of carrier cells and subsequent engraftment, have been established as more useful approaches with clear impact on functional brain neuroprotection (Fisher & Ray, 1994; Gage *et al.*, 1995; Snyder & Macklis, 1995; Martínez-Serrano & Björklund, 1996a; Martínez-Serrano & Björklund, 1997). For example, NGF-secreting fibroblasts, baby hamster kidney cells or neural progenitor cells are able to rescue basal forebrain cholinergic neurons after various lesions or reverse age-dependent atrophy of these neurons, improving cognitive function (Strömberg *et al.*, 1990; Dekker *et al.*, 1994; Winn *et al.*, 1994; Chen & Gage, 1995; Martínez-Serrano *et al.*, 1995a, b).

Medium-sized spiny projection neurons constitute the vast majority of neurons in the adult striatum (Heimer *et al.*, 1995). These neurons,

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most of which are GABAergic, are particularly susceptible to cell death induced by cerebral ischaemia (Pulsinelli *et al.*, 1982; Pulsinelli, 1985; Goto *et al.*, 1993) and excitotoxins (Boegman *et al.*, 1987; Davies & Roberts, 1988; Beal *et al.*, 1989; Forloni *et al.*, 1992; Figueredo-Cardenas *et al.*, 1994). Striatal projection neurons express the functional high-affinity receptors for BDNF and NT-3, i.e., TrkB and TrkC, respectively, but not the NGF receptor, TrkA, or the low-affinity neurotrophin receptor, $p75^{NTR}$ (Merlio *et al.*, 1993; Sobreviela *et al.*, 1994). Despite the lack of the functional receptors for this neurotrophin, biologically delivered NGF has been reported to counteract the death of striatal projection neurons caused by excitotoxic insults. Thus, Schumacher, Frim and collaborators (Schumacher *et al.*, 1991; Frim *et al.*, 1993a, b) initially described that intrastriatal grafts of fibroblasts, genetically modified to produce NGF, ameliorated the effects of quinolinic acid lesions in the rat striatum. Their findings were subsequently confirmed and extended using immortalized neural stem cells or epidermal growth factor-responsive stem cells as the carrier of the NGF gene (Martínez-Serrano & Björklund, 1996b; Kordower *et al.*, 1997). These studies have demonstrated that biologically delivered NGF can have generalized neuroprotective effects in the striatum, even on projection neurons not expressing NGF receptors at detectable levels.

The main objective of the present study was to explore the possibility that NGF, secreted by grafts of *ex vivo* transduced neural stem cells, could counteract the death of striatal projection neurons caused by transient focal ischaemia. We used immunocytochemical techniques in combination with stereological procedures to quantify the loss of these neurons at 48 h after 30 min of middle cerebral artery occlusion (MCAO). This ischaemic insult was chosen because the brain damage is largely restricted to the lateral striatum, and cerebral cortex shows much less neuronal death (Memezawa *et al.*, 1992b; Kokaia *et al.*, 1998). It is conceivable that the MCAO elicits both apoptotic and necrotic death (Linnik *et al.*, 1993; Li *et al.*, 1995; Charriaut-Marlangue *et al.*, 1996; Du *et al.*, 1996), with apoptosis localized to the penumbra area and necrosis predominating in the ischaemic core (Charriaut-Marlangue *et al.*, 1996). Grafting was performed 1 week prior to MCAO to allow for migration of the NGF-secreting cells into the striatal parenchyma (Lundberg *et al.*, 1997) and to avoid that the surgical procedure *per se* or disruption of the blood–brain barrier would influence the extent of the ischaemic lesion.

Materials and methods

Animals and experimental design

Twenty-eight adult, male Wistar rats (Møllegaard's Breeding Centre, Copenhagen, Denmark) weighing 267–325 g at the time of MCAO were used. The rats were housed under 12 h light/12 h dark conditions with *ad libitum* access to food and water. One week prior to the ischaemic insult (Fig. 1), the animals were randomly allocated to three experimental groups and either injected with vehicle ($n = 8$) or grafted with control-HiB5 cells ($n = 10$) or NGF-HiB5 cells ($n = 10$) unilaterally in the striatum. After fasting overnight with free access to water, all animals were subjected to MCAO for 30 min. The rats were killed 48 h after the ischaemic insult.

Culture of neural stem cell lines and ex vivo NGF gene transfer

The generation and characterization of the NGF-secreting and control cell lines used in the present experiment (clones E8 and D11, respectively) have been described in detail previously (Martínez-Serrano *et al.*, 1995b). The parental, conditionally immortalized, neural stem cell line was the E16 rat hippocampus-derived HiB5 cell line (Renfranz *et al.*, 1991), modified to produce and release mouse NGF by retroviral transduction. Cells were expanded at the permissive temperature for the immortalizing protein (+ 33 °C, tsA58/U19 mutant allele of the SV40 large T-antigen) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies AB, Sweden), supplemented with 10% foetal bovine serum, 2 mM glutamine, and 10 000 units/mL streptomycin and 10 000 units/mL penicillin. Prior to transplantation, the cells were labelled in culture for 72 h with ^{3}H-thymidine 10 μCi/mL (Amersham). For grafting, a single cell suspension with 150 000 cells/μL was prepared in Hank's balanced salt solution (Gibco) by trypsinization of nearly confluent monolayers.

Cell transplantation

To test whether neural stem cells of Sprague–Dawley origin (as the HiB5 derivatives used here) can survive transplantation into Wistar rats without immunosuppression, a separate group of three male Wistar rats (300 g body weight) were grafted with control cells in the right and NGF cells in the left striatum. The rats were anaesthetized with Equitesin (3 mL/kg i.p.), fixed in a Kopf stereotaxic frame and 1 μL of suspension containing ≈ 100 000 cells was then injected per side using a 10 μL Hamilton syringe. Coordinates with tooth bar at –2.3 mm below the interaural line were AP = 0.2, L = 3.5, V = 5.5. [AP, anterior or posterior to bregma; L, lateral to midline; V, vertical from dura according to the atlas of Paxinos and Watson (Paxinos & Watson, 1997), all distances in (mm).] These animals, which were not subjected to MCAO, were killed by transcardial perfusion of 4% paraformaldehyde after one week and then processed for ^{3}H-thymidine autoradiography (see below).

The other animals received NGF-producing or control cells or vehicle at three injection sites with two deposits at each site in the right striatum. The coordinates were with tooth bar set at –2.3 mm below the interaural line. Injection site 1: AP = 1.5, L = 2.5, V = 5.5 and 4.5; injection site 2: AP = 0.2, L = 3.0, V = 6.5 and 5.5; injection site 3: AP = –0.9, L = 4.2, V = 6.5 and 5.5. One microlitre of cell suspension was injected at each deposit (2 μL per injection site), the total number of cells per animal being about 900 000.

Middle cerebral artery occlusion

Anaesthesia was induced by inhalation of 3.5% halothane in $N_2O : O_2$ (70 : 30). The animals were intubated and then artificially ventilated with 1–1.5% halothane using a small respirator. A polyethylene catheter was inserted into the tail artery for sampling and blood pressure recording. Arterial blood pressure and body temperature were monitored using MacLab data acquisition system (AD Instruments, Australia). During surgery, ventilation was adjusted according to pO_2, pCO_2 and pH values. Physiological parameters, as measured before occlusion, are presented in Table 1. The MCAO was performed according to the technique described by Koizumi *et al.* (1986) and modified by Zhao *et al.* (Zhao *et al.*, 1994; Kokaia *et al.*, 1995). In brief, the right common, internal and external carotid arteries were exposed and the external carotid artery was ligated. The common carotid artery was closed by a ligature, and the internal carotid artery was temporarily closed by a microvascular clip. To prevent thrombosis, 30 IE heparin was given intra-arterially. A filament made from a monofilament fishing line (Stren, supertough, 0.25 mm diameter, Du Pont, Wilmington, DE, USA) with a melted tip (0.28 mm diameter) and a distal cylinder of silicon rubber (Silastic E, Dow Corning, MI, USA) was inserted into the internal carotid artery through the common carotid artery and advanced to block the blood flow in the middle

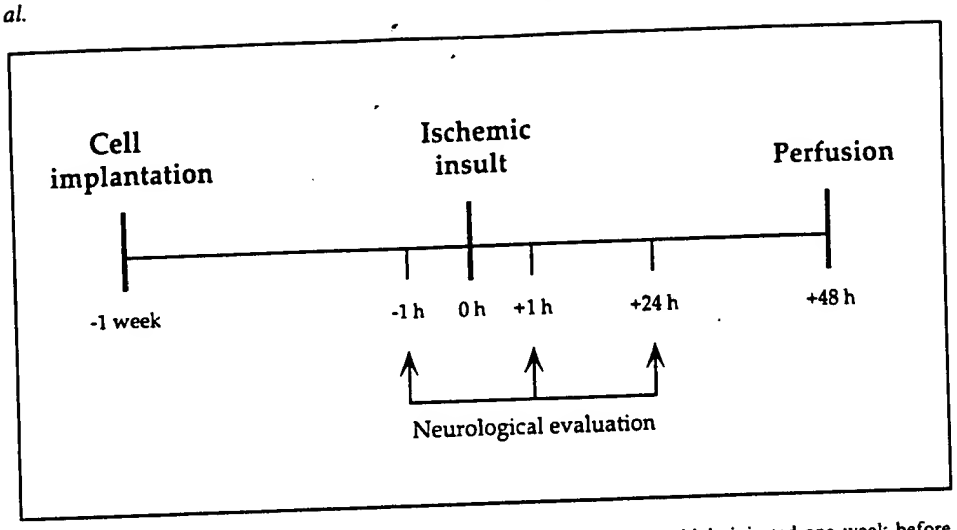

FIG. 1. Experimental design. The rats were either grafted with NGF-producing or control cells, or vehicle-injected one week before the focal ischaemic insult, which was produced by 30 min middle cerebral artery occlusion (MCAO). All animals were perfused for immunocytochemistry 48 h thereafter. Neurological assessment was carried out 1 h before and 1 and 24 h after MCAO.

TABLE 1. Physiological parameters and neurological scores in rats subjected to middle cerebral artery occlusion

	Physiological parameters							Neurological scores		
Group	Weight (g)	Glucose (mM)	pH	pCO_2 (mmHg)	pO_2 (mmHg)	Temp. (°C)	Blood pressure (mmHg)	Before MCAO	1 h after MCAO	24 h after MCAO
Vehicle-inj.	298 ± 6	3.8 ± 0.3	7.43 ± 0.01	36.8 ± 1.1	108.8 ± 3.8	37.0 ± 0.1	110.0 ± 3.4	5.0 ± 0.0	1.0 ± 0.2	4.6 ± 0.3
NGF-graft	303 ± 4	5.0 ± 0.4	7.46 ± 0.01	33.9 ± 0.8	118.8 ± 3.3	37.1 ± 0.1	117.4 ± 2.3	4.4 ± 0.3	1.6 ± 0.4	4.2 ± 0.3
Control-graft	301 ± 7	5.0 ± 0.3	7.45 ± 0.01	36.2 ± 0.8	112.9 ± 4.4	37.3 ± 0.1	117.0 ± 4.5	4.8 ± 0.2	2.0 ± 0.4	4.6 ± 0.3

Physiological parameters (except body weight) were measured after the surgical procedures, just before insertion of the occluding filament. The neurological assessment comprised scoring of the placing of the fore- and hindlimb contralateral to the MCAO and of circling behaviour. Score = 5 indicates no deficit and 0 maximum impairment. Data are means ± SEM. There were no significant differences between the groups ($P > 0.05$, one-way ANOVA followed by Bonferroni–Dunn *post-hoc* test).

cerebral artery. When the surgical procedure had been completed, anaesthesia was discontinued and the rat was awake after 10–15 min. Reperfusion was started after an occlusion time of 30 min by withdrawal of the filament under brief anaesthesia using halothane (1.5%) in $N_2O : O_2$ (70 : 30). The MCAO was carried out blindly, i.e., without knowing which experimental group the animals belonged to.

Neurological evaluation

Neurological examination was carried out blindly just before and at 1 and 24 h after MCAO (Fig. 1). The main aim of this analysis was to provide a measure of successful MCAO and recirculation. Fore- and hindlimb placing was assessed mainly according to De Ryck *et al.* (1989), and circling towards the paretic side was evaluated as previously described by Bederson *et al.* (1986).

The placing of the fore- and hindlimb on the side contralateral to MCAO, was examined when the limb, gently pulled down and away from a table edge, suddenly was released. To evaluate hindlimb placing, the left side was perpendicular to the edge of a table and for forelimb placing the forepaws were perpendicular to the table edge with head and whiskers outside the edge. The placing of each limb was graded as 'no' placing (score 0), 'incomplete' and/or 'delayed' (> 2 s) placing (score 1), and 'correct' placing (score 2). Circling was evaluated when the rat was allowed to move freely in an open area. Circling to the non-occluded side was scored as 0 and no circling as 1. The scores for fore- and hindlimb placing and circling behaviour were summed up for each animal (maximum score = 5). Animals which were fully awake and had lost one grade or more in the neurological scoring at 1 h after MCAO were included for further analyses.

Immunocytochemistry

Animals were deeply anaesthetized with chloral hydrate (400 mg/kg) and transcardially perfused with physiological saline followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, postfixed with the same fixative overnight and equilibrated in 30% sucrose. Series of 40-µm-thick sections were taken through the striatum, and stored cryoprotected at –20 °C until use. For free-floating immunocytochemical stainings, the sections were rinsed and endogenous peroxidase quenched in 3% H_2O_2. After blocking in appropriate serum, the sections were incubated overnight (for ChAT, incubation with the primary antibody was for 72 h at 4 °C) with the primary mouse monoclonal antibody. After rinsing, sections were incubated with the appropriate biotinylated-secondary antibody (horse antimouse), reacted with ABC Kit (Vector, Burlingame, USA), and peroxidase was then developed in a nickel-intensified DAB reaction. Antibody titres and sources were as follows: against dopamine- and adenosine 3′:5′-monophosphate-regulated phosphoprotein with a molecular weight of 32 kDa (DARPP-32, marker for striatal projection neurons) 1 : 20000, a gift from Dr P. Greengard, Rockefeller University, New York, NY, USA, against neuronal-specific antigen (NeuN, marker for postmitotic neurons) 1 : 100, a gift from Dr R. J. Mullen, University of Utah, Salt Lake City, UT, USA, and against choline

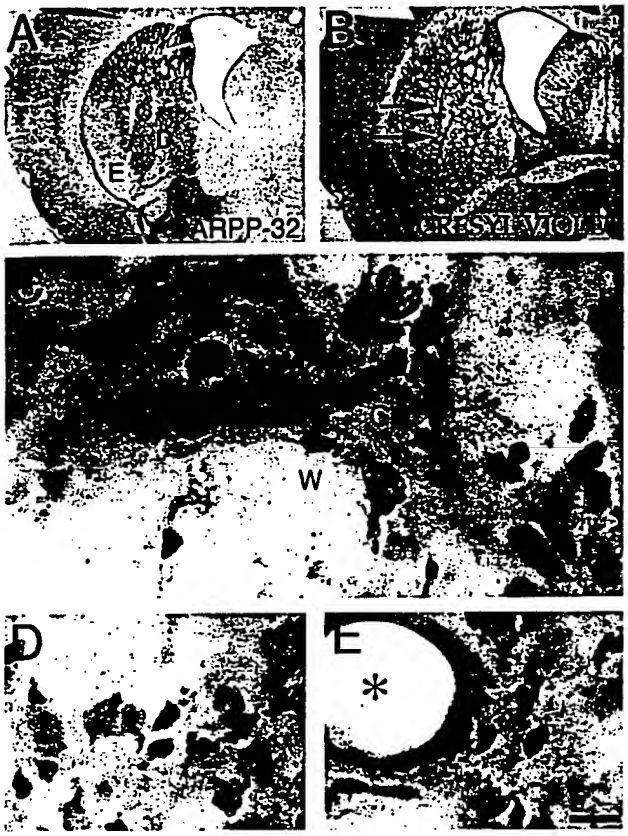

FIG. 2. Survival and integration of transplanted cells. Adjacent sections immunostained for DARPP-32 (A) or subjected to ^{3}H-thymidine autoradiography and counterstained with cresyl violet (B), to illustrate graft survival at different locations and lesion severity. Arrows in B depict one implantation site. The location of the higher magnification photomicrographs in C–E is indicated in A. Grafted cells (identified by silver grains on the autoradiogram) are illustrated in regions with minor (C), intermediate (D) and severe ischaemic damage (E). Note the preferential distribution of the cells in grey matter areas (G). The asterisk in E denotes a blood vessel. W, white matter. Scale bar = 1.5 mm in A and B and 18 μm in C–E.

acetyltransferase (ChAT, marker for cholinergic neurons) 1 : 1000, Chemicon.

^{3}H-Thymidine autoradiography

One series of sections from each of the grafted animals was mounted on slides, dehydrated and delipidated before immersion in autoradiographic emulsion (K5, Ilford). The sections were left for 5 weeks at −20 °C in the dark, and then developed. Before coverslipping, the specimens were counterstained with cresyl violet.

Morphometric analysis

The number of DARPP-32- and NeuN-immunopositive neurons was quantified using stereological procedures, which allow for unbiased estimates of cell numbers within a defined brain structure (Gundersen *et al.*, 1988). Analyses were performed using the Computer Assisted Stereological Toolbox (CAST)–GRID software (Olympus, DK A/S, Albertslund, Denmark), controlling an X-Y-Z motorized Olympus BH-2 microscope stage. Images were first acquired with a CCD-IRIS colour video camera and the borders of the striatum were marked at small magnification (×4) using the computer mouse. Cells were then counted at ×40 magnification in randomly selected fields chosen by the computer controlling the stage.

DARPP-32- and NeuN-immunoreactive neurons were first quantified in the vehicle-injected, control-cell- and NGF-cell-grafted striata at four representative coronal levels through the striatum: 1, AP 1.7 mm; 2, AP 0.7 mm; 3, AP −0.3 mm; 4, AP −1.4. mm. In six randomly selected animals, the non-lesioned side was analysed similarly in order to obtain cell counts from the intact striatum. Using image analysis (CAST–GRID software), measurements of the whole striatum and the area devoid of DARPP-32 immunostaining were made at the same levels and the remaining, non-lesioned area, was calculated thereafter. In a separate analysis, the number of DARPP-32-positive neurons was counted stereologically in 17 sections spaced at 320-μm intervals throughout the striatum (from 2.0 mm rostral to 3.3 mm caudal to bregma) in animals receiving control or NGF-secreting transplants. Due to their lower numbers, the ChAT-positive cholinergic interneurons were counted manually and blindly at the four coronal levels defined above, using ×10 magnification.

Statistical analysis

Evaluation of differences in the number of immunopositive cells and in the area with remaining DARPP-32 immunoreactivity between the intact striatum and the lesioned striatum in the three experimental groups, as well as differences in neurological scores and physiological parameters were performed using one-way analysis of variance (ANOVA) followed by the indicated *post-hoc* tests. Numbers of DARPP-32 positive neurons in sections throughout the striatum from control and NGF-cell grafted rats were compared using one-tailed unpaired Student's *t*-test. To compare numbers of ChAT-positive neurons between the striatum ipsilateral and contralateral to MCAO, paired Student's *t*-test was used. Significance was set at $P < 0.05$.

Results

Physiological parameters and neurological assessment

Table 1 shows physiological parameters as measured 1 week postgrafting and just before the induction of ischaemia. There were no significant differences between the experimental groups. The neurological assessment performed 1 h before MCAO revealed a mild impairment in two animals with control-cell grafts and in three animals with NGF-secreting grafts. These rats exhibited a slight deficit in hindlimb placing, which suggests that the surgical procedure used here may cause a minor brain damage in some rats. However, there were no significant differences in neurological scores between the groups. When the rats were evaluated at 1 h after MCAO, marked neurological impairment was observed in the majority of animals. In four animals (two in the control-cell group, one in the NGF-cell group and one in the vehicle-injected group), the MCAO did not result in any deficit, indicating unsuccessful occlusion. These animals were not included in the further analysis. The other rats exhibited circling behaviour to the non-occluded side and showed no placing of the hindlimb. The placing of the forelimb was delayed or incorrect. The neurological scores in these remaining animals (seven vehicle-injected, eight control-cell grafted and nine NGF-cell grafted) did not differ between the groups. At 24 h after MCAO, there was marked behavioural recovery in all animals, without any significant differences between the groups (Table 1).

Survival of grafted cells

The focal ischaemia model used here has been extensively characterized in Wistar rats (Memezawa *et al.*, 1992a,b; Zhao *et al.*, 1994; Kokaia *et al.*, 1995), and we therefore performed all experiments on animals of this strain. Because the HiB5 cells and their derivatives

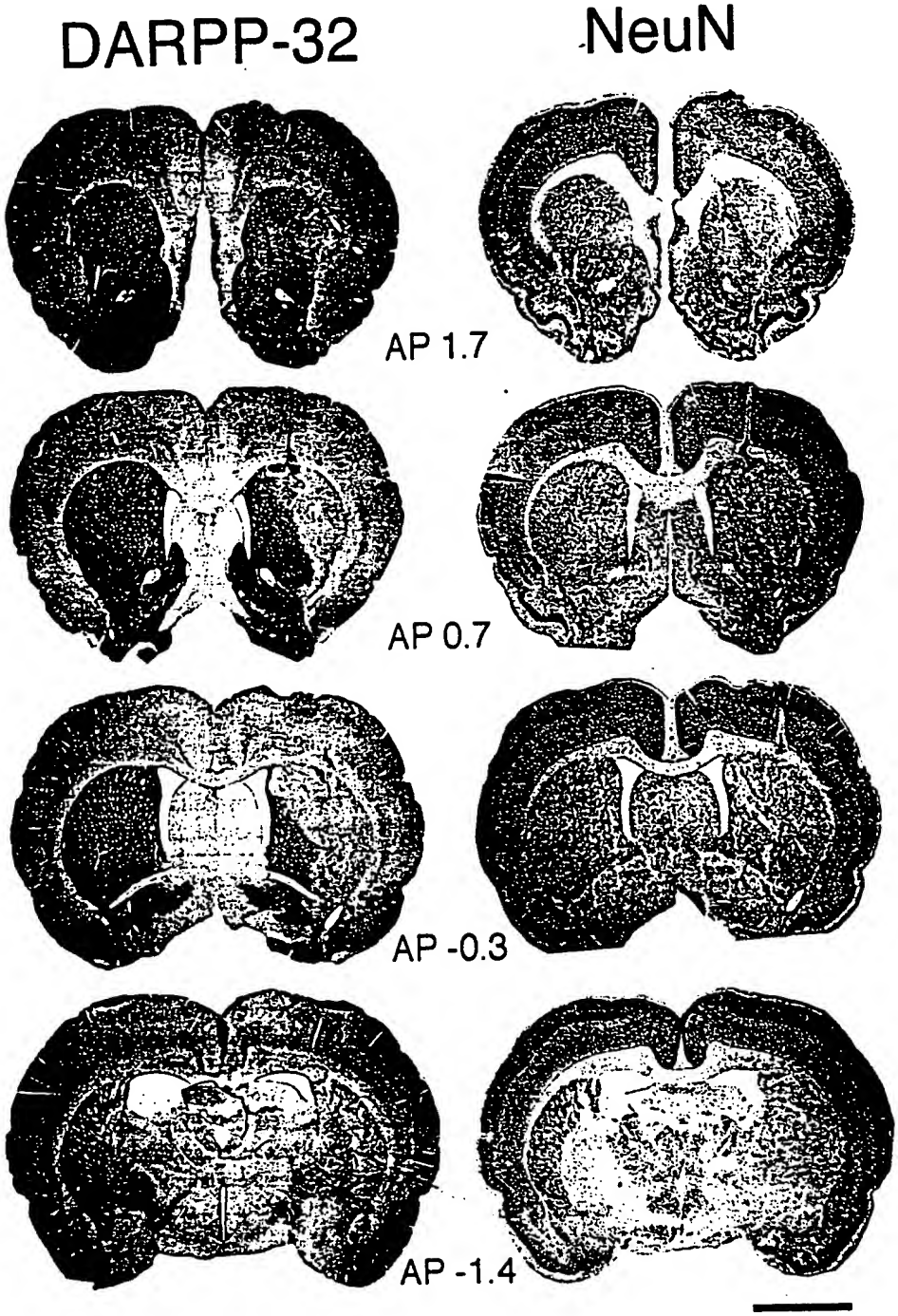

FIG. 3. Overview of ischaemic damage. DARPP-32- and NeuN-immunostained sections from a vehicle-injected animal subjected to 30 min of MCAO. The extent of the lesion is illustrated at four coronal levels distributed throughout the striatum. These levels are marked according to their relative position to bregma (in mm) according to the atlas of Paxinos and Watson (Paxinos & Watson, 1997). Scale bar = 3 mm.

were generated from Sprague–Dawley rats, we first explored whether intrastriatal grafts of these cells can survive in Wistar rats without immunosuppressive treatment. The use of cyclosporin seemed unfavourable in the present experiment due to the reported protective action of this drug against ischaemic damage (Uchino *et al.*, 1995). Grafted cells were identified using autoradiography combined with cresyl violet staining to locate the nuclei of cells labelled with ^{3}H-thymidine in vitro prior to transplantation. The survival and distribution of the grafted control and NGF-secreting HiB5 cells in Wistar rats, not subjected to ischaemia, closely resembled what has previously been described in Sprague–Dawley rats for these and the parental HiB5 cell lines (data not shown; Martínez-Serrano *et al.*, 1995b; Lundberg *et al.*, 1997). The grafted cells had migrated 1–1.5 mm away from the implantation site and seemed to be well integrated into the host striatum. No evidence for immunological rejection, e.g. lymphocyte infiltration or tissue damage, was obtained.

Also in the two grafted groups of rats, which had been subjected to 30 min of MCAO, the transplants did not induce any noticeable

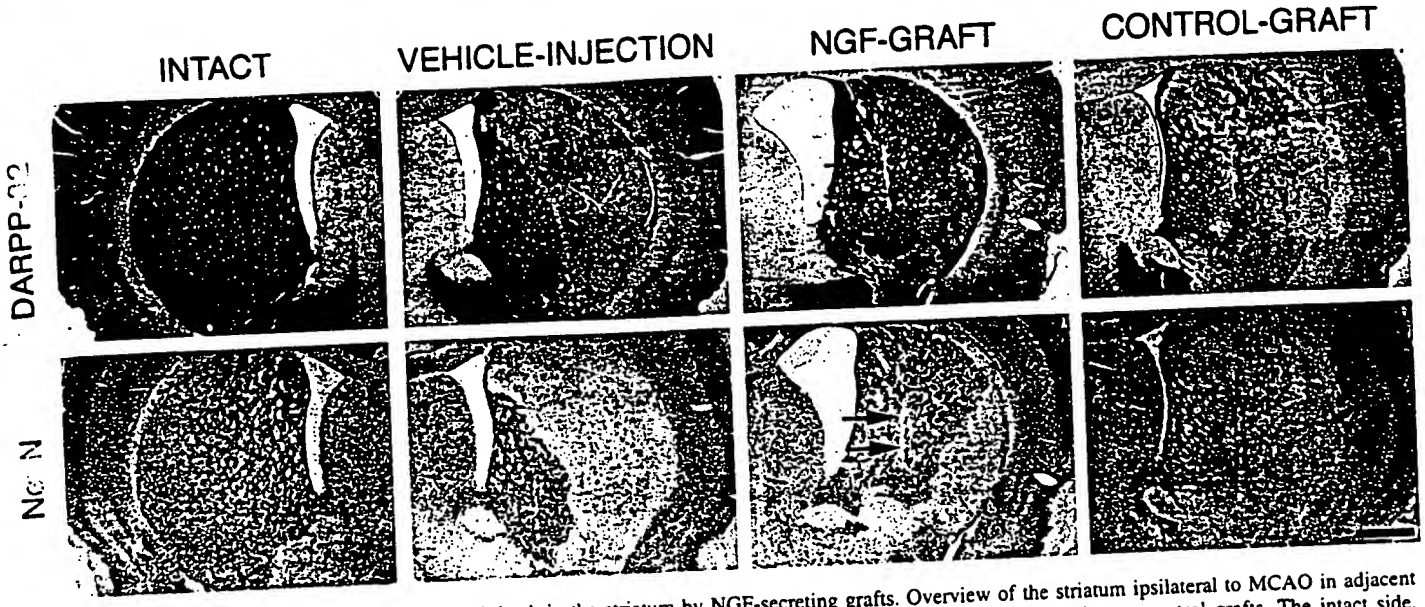

FIG. 4. Protection against ischaemia-induced neuronal death in the striatum by NGF-secreting grafts. Overview of the striatum ipsilateral to MCAO in adjacent sections immunostained for DARPP-32 or NeuN, taken from a vehicle-injected rat and from animals with NGF-secreting or control grafts. The intact side, contralateral to MCAO is also illustrated. Note the marked reduction of the ischaemic damage in the NGF-cell treated animal, as compared with both vehicle-injected and control-cell grafted rats. The location of one of the grafts is depicted by arrows. Scale bar = 1 mm.

disruption of the striatal cytoarchitecture (Fig. 2A,B). This was also the case in the animals exhibiting mild neurological impairment at the assessment prior to the MCAO. However, these rats showed small cortical lesions located close to the needle tracts. Surviving grafted cells could be detected in the striatum of all transplanted animals. As illustrated in Fig. 2, the survival and distribution pattern of the grafted cells were dependent both on their location in the striatum and the severity of the ischaemic lesion in that particular area, as determined by the loss of DARPP-32-immunoreactive neurons. In regions of intermediate-to-low ischaemic damage (Fig. 2C,D), and relatively close to the implant site, groups of grafted cells could be easily identified, preferably distributed in grey matter zones and intermingled with host neurons and glia. The histological appearance of the grafted cells, with a small cell body size and glia-like morphology, was the same as that observed in other studies involving intrastriatal transplantation of the same cell lines (Martínez-Serrano *et al.*, 1995b; Martínez-Serrano & Björklund, 1996b; Lundberg *et al.*, 1997). In regions with severe ischaemic damage, grafted cells could only be found close to the implantation site. The grafted cells illustrated in Fig. 2(E) were located in the lateral striatum, in an area with a severe ischaemic lesion. It seems possible that these cells may have survived due to their close proximity to a blood vessel.

Graft effects on ischaemic damage

The extent of the ischaemic lesion in a representative, vehicle-injected animal as observed in DARPP-32- and NeuN-immunostained sections, is illustrated in Fig. 3 at four coronal levels. The lesion, defined as the area with lost immunoreactivity for DARPP-32 and NeuN, was located in the dorsolateral striatum except most caudally, where the entire cross-section of this structure was affected. The loss of immunoreactivity for DARPP-32 and NeuN closely matched each other. The characteristics and extent of the lesion did not differ from what has been observed following 30 min of MCAO in animals not subjected to vehicle injection or grafting (Memezawa *et al.*, 1992b; Kokaia *et al.*, 1998).

The distribution of the ischaemic damage was uniform among the vehicle-injected animals, and the borders between lesioned and non-lesioned areas were sharp (Figs 3 and 4). Implantation of non-NGF-producing control cells caused more variability in the extent of the lesion. In four out of eight animals in this group the lesion had a patchy appearance, as compared with one out of seven rats in the vehicle-injected group. The boundaries of the lesion were more difficult to define. Also in the group treated with NGF-secreting cells, the extent of the lesion varied between rats and in five out of nine animals had a patchy appearance. Two animals implanted with NGF-cells showed ischaemic damage similar to that in the vehicle-injected group. Interestingly, considerably fewer surviving grafted cells could be detected in these animals as compared with the other rats in this group. The majority of animals with NGF-grafts exhibited little or no ischaemic damage near the implantation sites, identified by the needle tracts, and in the rostral and dorsal parts of the striatum. Also in the ischaemic core, i.e. most caudally and laterally in the striatum, the lesion appeared to be less severe as compared with the vehicle-injected and control-cell grafted groups, with areas showing nearly normal DARPP-32 and NeuN immunostaining.

The number of striatal neurons immunoreactive for DARPP-32 or NeuN was first quantified using stereological procedures at 4 predetermined levels (illustrated in Fig. 3) on the side of MCAO in all rats given vehicle injections or grafted with NGF-secreting or control cells, as well as on the contralateral side in randomly selected animals from all groups (Fig. 5). As observed with both DARPP-32 and NeuN immunocytochemistry, the neuronal loss at 48 h following 30 min of MCAO was most severe in the caudal striatum. Compared with the intact side, the reduction of DARPP-32 and NeuN-immunopositive neurons at the most caudal striatal level in vehicle-injected animals amounted to 95 and 90%, respectively. The corresponding figures at the most rostral level were 62 and 61%. There were no significant differences between vehicle-injected and control-cell grafted striata in the number of DARPP-32- or NeuN-stained neurons, except most rostrally, where more NeuN- but not DARPP-32-positive neurons were found in the striatum implanted with control cells. In contrast, the number of both DARPP-32- and NeuN-immunopositive neurons in the striatum grafted with NGF-producing cells was found to be significantly higher as compared with both vehicle-injected (at

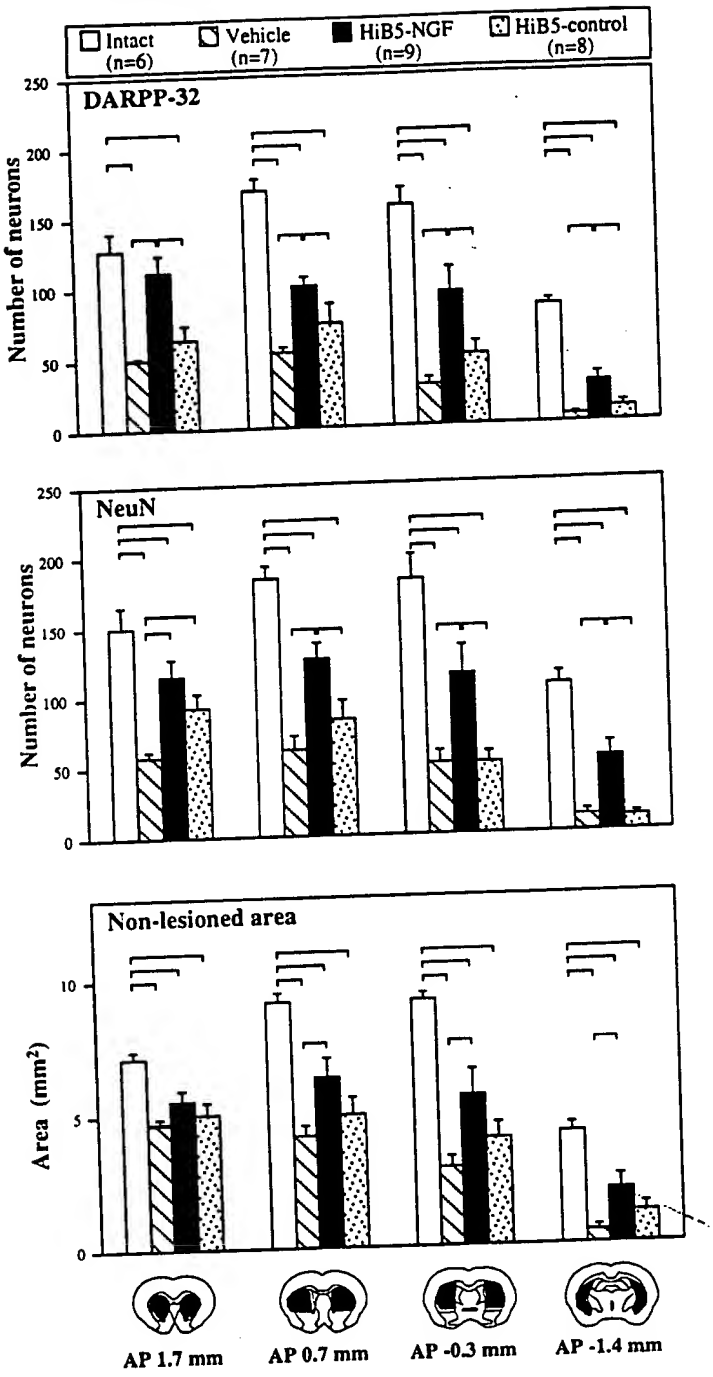

FIG. 5. Quantitative analysis of DARPP-32- and NeuN-immunoreactive neurons and non-lesioned striatal area after MCAO. Data are from intact (Intact) striatum, contralateral to MCAO, and from ipsilateral striatum in animals with either vehicle injections (Vehicle), or grafted with NGF- (HiB5-NGF) or control-cells (HiB5-control). Number of neurons was counted using stereological procedures and area of DARPP-32-positive striatal staining ipsilateral to MCAO was measured with computerized image analysis at the four levels illustrated in Fig. 3. Significant differences between groups are indicated by lines above the bars ($P < 0.05$, one-way analysis of variance (ANOVA), followed by Fisher PLSD *post-hoc* test).

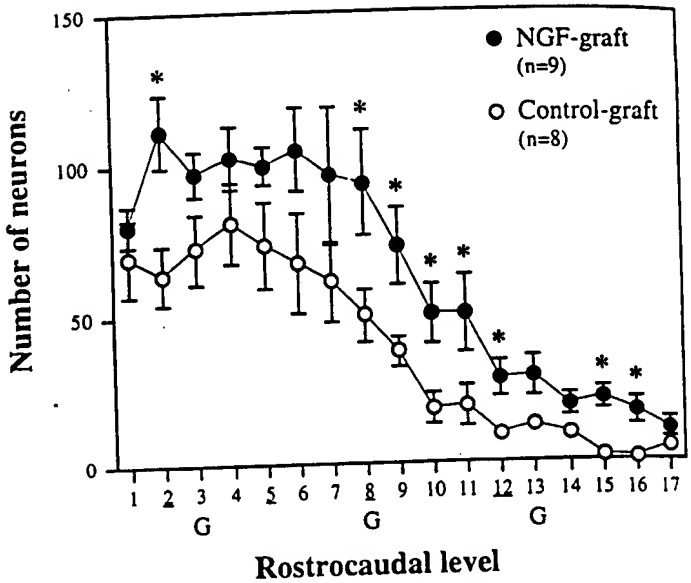

FIG. 6. Rostrocaudal distribution of the neuroprotective effect induced by NGF-secreting cells. DARPP-32-immunostained striatal neurons were counted in every section, spaced at 320-μm interval, throughout the striatum, in the rats with control- or NGF-secreting grafts, 48 h after 30 min of MCAO. G depicts approximate graft locations. Level 1 and 17 correspond to +2.0 mm and −3.3 mm, in relation to bregma, respectively (Paxinos & Watson, 1997). Underlined levels are those illustrated in Fig. 3. *, $P < 0.05$, unpaired Student's *t*-test.

all four levels) and control-cell transplanted striatum (all levels with DARPP-32, three caudal levels with NeuN). The implantation of NGF-synthesizing cells was estimated to prevent 45% (mean of data from the four levels, range 26–72%) and 46% (range 40–56%) of the loss of DARPP-32- and NeuN-positive neurons, respectively, observed in the vehicle-injected striatum.

Similar to cell counting, measurement of the non-lesioned area (Fig. 5) showed more pronounced ischaemic damage at caudal as compared with rostral striatal levels (89 and 35% reduction at most caudal and rostral levels, respectively, in vehicle-injected animals). Furthermore, this area was significantly larger in NGF-cell-grafted as compared with vehicle-injected striata, except at the most rostral level. However, the difference between striata with NGF-producing cells and those with control cells did not reach statistical significance.

Because the cell deposits were placed at discrete locations, it seemed possible that they had induced only local neuroprotective effects. Therefore, the number of DARPP-32-immunoreactive neurons was also quantified in regularly spaced sections throughout the entire striatum of animals receiving control- or NGF-cell transplants. Animals receiving NGF-secreting cells had significantly more DARPP-32-immunoreactive neurons as compared with rats with control cells at almost every level in the caudal half of the striatum (approximately twice the number of neurons) (Fig. 6). Also at more rostral levels, the mean number of neurons was higher in every section in the rats with NGF-grafts. However, due to higher variability between animals in the severity of the ischaemic lesion in the most rostral striatum, this difference only reached statistical significance at one level.

We also explored the possibility that the larger number of neurons in the striatum with NGF-producing grafts could be due to reversal of a possible ischaemia-induced down-regulation of the studied protein markers instead of reflecting neuronal rescue. However, in all animal groups, the boundaries of the region identified by the loss of DARPP-32 immunostained neurons exactly overlapped with the locations where unequivocal signs of dead neurons (with shrunk, condensed and darkly stained cell bodies) could be observed in paired cresyl

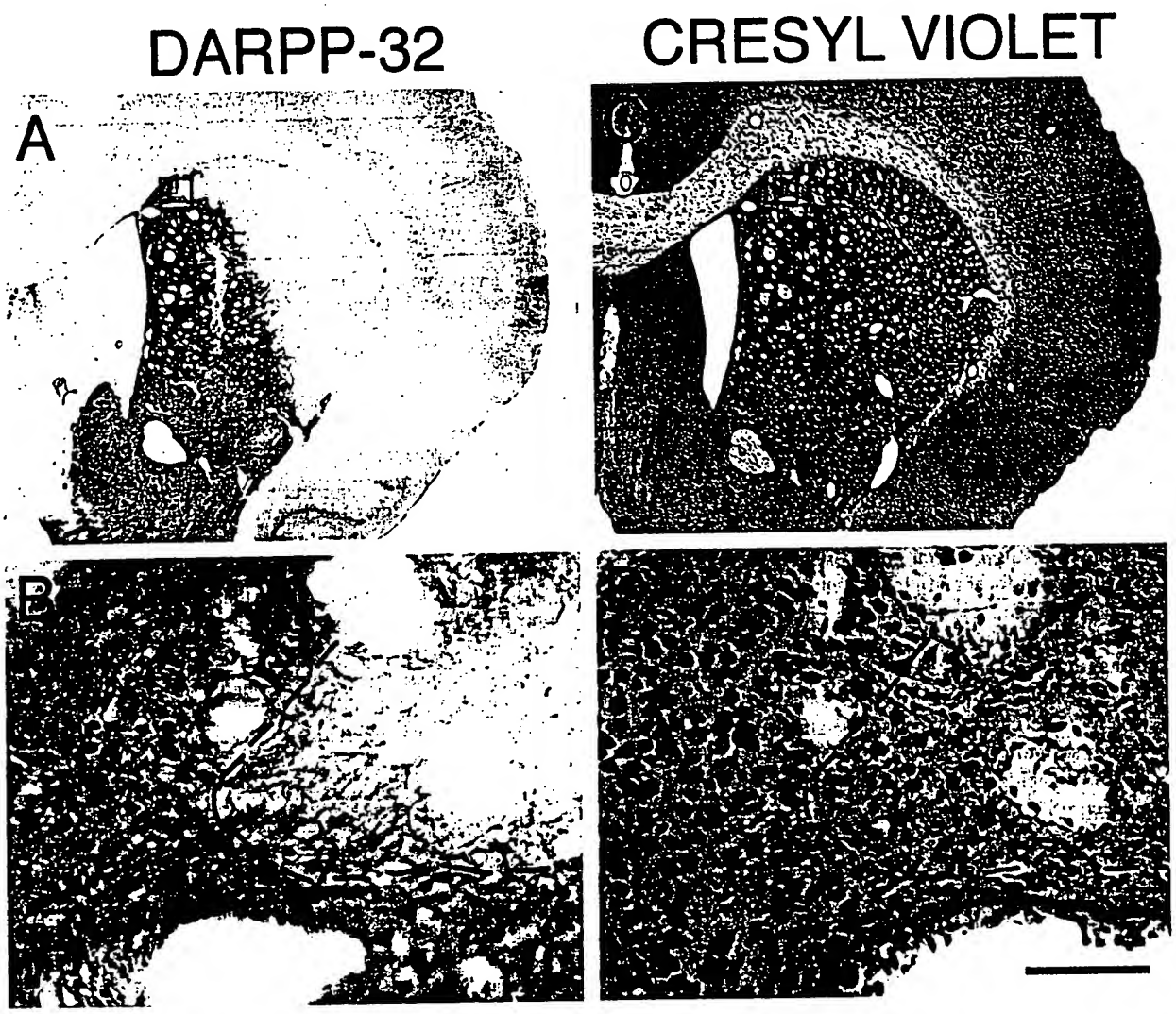

FIG. 7. Demonstration of ischaemic neuronal death. Photomicrographs of a DARPP-32 immunostained section (A,B), and of an adjacent section, stained with cresyl violet (C,D) through the striatum of a rat grafted with control-cells and then subjected to 30 min of MCAO. B and D show the framed areas in A and C, respectively, in higher magnification. There is a close correspondence between the area with loss of DARPP-32 immunostained neurons (outlined by a broken line in B), and the area containing shrunk, pycnotic neurons as revealed by cresyl violet-staining (outlined in D). Scale bar = 1.5 mm in A and C and 75 μm in B and D.

violet-stained sections (Fig. 7). This strongly supports the view that the lack of DARPP-32 immunoreactivity induced by the present insult signifies neuronal death.

In contrast to the marked loss of DARPP-32-positive projection neurons, we observed no significant change of the number of ChAT-positive cholinergic interneurons in the vehicle-injected or control-cell grafted striatum following 30 min of MCAO (Table 2). The NGF-cell-grafted striatum, on the other hand, showed significantly more neurons with detectable levels of ChAT as compared with the intact, contralateral striatum at the three rostral, representative levels (81, 65 and 41% increase, respectively; Table 2).

Discussion

The present results indicate that intrastriatal implantation of cells genetically engineered to secrete NGF can ameliorate neuronal death in the rat striatum at 48 h following 30 min of MCAO. The combination of stereological or manual quantification of cell numbers and specific neuronal phenotypic markers used here has allowed, for the first time, characterization of the ischaemic damage and the protective effects at the cellular level. We found degeneration of the majority of DARPP-32-immunopositive projection neurons, whereas ChAT-positive cholinergic interneurons were resistant to the ischaemic insult (Kokaia *et al.*, 1998). The NGF-secreting grafts reduced the loss of striatal projection neurons by about 45%.

In most previous studies on focal ischaemia models, the identification of infarcted and surviving tissue has been carried out at the macroscopic level, often by the use of vital stains (see for example Bederson *et al.*, 1986; Wang *et al.*, 1997). The discrepancy observed here between area measurements and unbiased, quantitative assessment of neuronal numbers indicates that procedures using macroscopic analysis of vital staining may not reveal all information that can be obtained in the present type of experiment. Quantitative cellular data combined with various neuronal markers seem highly warranted, in particular, after less severe ischaemic insults, in which selective neuronal death is a prominent feature (Memezawa *et al.*, 1992b), and in the penumbra area and other transition zones, e.g. close to a trophic factor-secreting graft.

TABLE 2. Number of ChAT-positive neurons in the striatum ipsi- and contralateral to vehicle injection, or control-cell or NGF-cell grafts in rats subjected to middle cerebral artery occlusion

	AP 1.7 mm		AP 0.7 mm		AP –0.3 mm		AP –1.4 mm	
Group	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
Vehicle-inj.	57.9 ± 11.9	51.7 ± 11.3	72.0 ± 11.4	51.9 ± 11.1	95.1 ± 9.8	71.1 ± 7.1	46.6 ± 6.0	34.4 ± 5.2
NGF-graft	91.3 ± 10.0*	50.4 ± 6.6	88.3 ± 6.5*	53.7 ± 7.9	85.9 ± 5.4*	61.1 ± 3.8	42.0 ± 8.3	33.8 ± 6.0
Control-graft	78.9 ± 6.2	71.3 ± 10.7	94.5 ± 7.1	79.0 ± 16.6	93.0 ± 4.8	81.9 ± 16.2	37.8 ± 7.2	31.5 ± 6.6

Data are means ± SEM. Cells were quantified manually at four representative coronal levels throughout the striatum. AP, anterior or posterior to bregma according to the atlas of Paxinos & Watson (1997). *, significant difference compared to contralateral striatum. $P < 0.05$, Student's paired *t*-test.

In the present experiment, amelioration of neuronal death by the NGF grafts was demonstrated at 48 h after the ischaemic insult. It might be argued that the NGF treatment only delayed and not prevented the death of striatal projection neurons and therefore that the observed protective effect was only transient. Although this possibility cannot be excluded, it seems highly unlikely for several reasons. First, in contrast to cerebral cortex, which exhibits delayed neuronal death after both focal and global ischaemia, several studies have demonstrated that the majority of striatal neurons are damaged rapidly, and within 12–24 h, show evidence of cell death after ischaemic insults (Pulsinelli *et al.*, 1982; Garcia *et al.*, 1995; Wiessner *et al.*, 1996). For example, we observed (Z. Kokaia *et al.*, unpublished data) clear degenerative changes in virtually all projection neurons in the dorsolateral striatum between 6 and 16 h after 2 h of MCAO. Also arguing for a rapid cell loss in this region, we found no difference between 48 h and 1 week after 30 min of MCAO in the number of remaining striatal neurons (Kokaia *et al.*, 1998). Second, the present insult caused a major loss of striatal neurons (60–90%) which was markedly reduced by about 45% in animals with NGF-secreting grafts. In conclusion, there are no experimental data supporting a hypothetical scenario in which elevated striatal NGF levels would induce a large proportion of striatal neurons, normally otherwise degenerating within 24 h after the insult, to survive up to 48 h with normal cresyl-violet-, NeuN- and DARPP-32-staining and then die.

The properties of the cell line used as carrier for the transgene has been described in detail previously (Martínez-Serrano & Björklund, 1996a, 1997; Lundberg *et al.*, 1997). The release rate *in vitro* is 2 ng NGF/h/10^5 cells, and the cells are able to increase local NGF bioactivity and protein levels in transplanted brain regions up to 10 weeks postgrafting (Martínez-Serrano *et al.*, 1995a,b, 1996). Moreover, expression at the mRNA level persists even at 9 months following transplantation (Martínez-Serrano & Björklund, 1998). The NGF secretion from the present cell line is stable both in a dividing culture, after differentiation, and following intracerebral transplantation (Martínez-Serrano *et al.*, 1995b). The cells adopt a phenotype resembling resident glia and have migrated up to 1–1.5 mm away from the implantation site after 1 week. In the present experiment, surviving grafted cells with a similar distribution and morphology were detected in the striatum at 48 h following ischaemia. It seems likely therefore that elevated NGF levels were present in the striatum both at the time of MCAO and for 48 h thereafter. In support of this idea, significantly more neurons with detectable levels of ChAT were found in the striatum with NGF-cell grafts, as previously observed also in the excitotoxic model (Martínez-Serrano & Björklund, 1996b). This probably reflects increased synthesis of the enzyme triggered by NGF (Hagg *et al.*, 1989; Venero *et al.*, 1994; Martínez-Serrano & Björklund, 1996b).

Several lines of evidence support that the reduction of neuronal death in the striatum induced by 30 min of MCAO in the transplanted rats was due to the NGF secretion by the graft. First, no significant protective action was observed after either vehicle injection or implantation of non-NGF-producing control cells. Second, in the group of animals transplanted with NGF cells, the rats which showed virtually no reduction of ischaemic damage also exhibited very poor graft survival. Third, there were no systematic differences between the experimental groups in the severity of the ischaemic insult. Fourth, other studies have shown that NGF can ameliorate death of hippocampal CA1 neurons induced by transient global ischaemia, (Shigeno *et al.*, 1991; Pechan *et al.*, 1995).

The mechanisms by which NGF ameliorates ischaemic damage in the striatum remain to be elucidated. It is remarkable that the protective action is exerted on projection neurons, which lack both the high- and low-affinity receptors for NGF, i.e. TrkA and $p75^{NTR}$, respectively (Yan & Johnson, 1989; Sobreviela *et al.*, 1994). However, there is good evidence from several lesion models that NGF can have protective effects also on neurons not expressing NGF receptors. Death of striatal projection neurons following injection of excitotoxins in adult rats (Schumacher *et al.*, 1991; Frim *et al.*, 1993a, b; Emerich *et al.*, 1994; Martínez-Serrano & Björklund, 1996b; Kordower *et al.*, 1997) or after hypoxic-ischaemic insults in neonatal animals (Holtzman *et al.*, 1996) is mitigated by administration of NGF. Similarly, NGF protects hippocampal CA1 neurons against degeneration caused by transient forebrain ischaemia in gerbils (Shigeno *et al.*, 1991) and rats (Pechan *et al.*, 1995), despite the very low levels of TrkA expressed in these neurons (Cellerino, 1996). Our data do not reveal when, in relation to the insult, NGF has to be administered in order to increase the resistance of striatal projection neurons to ischaemic damage. Arguing in favour of an acute protective action (Holtzman *et al.*, 1996) found that NGF ameliorated neuronal injury in the neonatal striatum when injected intraventricularly just before and at 48 h after a hypoxic-ischaemic insult.

Hypothetically, NGF might counteract several of the molecular and cellular mechanisms believed to be involved in mediating ischaemic damage, including enhanced production of free radicals or nitric oxide, derangement of cell calcium homeostasis, and release of excitatory amino acids and activation of glutamate receptors. Supporting a role for NGF in activating free-radical detoxifying systems, NGF induced increased catalase levels in cultured PC12 cells (Sampath *et al.*, 1994), and implantation of NGF-producing fibroblasts into the rat striatum gave rise to elevated catalase activity (Frim *et al.*, 1994). The elevated striatal NGF levels might also counteract the neurotoxicity caused by nitric oxide, which is released during focal ischaemia and reperfusion (Malinski *et al.*, 1993; Kumura *et al.*, 1994; Sato *et al.*, 1994). Treatment with NGF for several days increases the resistance of PC12 cells to nitric oxide (Wada *et al.*, 1996). Furthermore, intrastriatal grafts of NGF-secreting fibroblasts attenuate 3-nitrotyrosine formation induced by the mitochondrial toxin 3-nitropropionic acid, possibly by inhibiting nitric oxide production (Galpern

et al., 1996). NGF has also been shown to prevent the increase of intracellular Ca^{2+} levels and the degeneration of hippocampal neurons exposed to hypoglycaemia *in vitro* (Cheng & Mattson 1991; Mattson & Cheng, 1993). Several studies have demonstrated that NGF can reduce excitotoxic neuronal damage in the striatum (Schumacher *et al.*, 1991; Frim *et al.*, 1993a, b; Emerich *et al.*, 1994; Martínez-Serrano & Björklund, 1996b). In addition, it cannot be excluded that NGF might have altered the pattern of striatal blood flow during and following the present insult. It is unlikely that the protective effect was due to a NGF-induced change of postischaemic temperature regulation. In a parallel study, with identical experimental groups, the animals were normothermic at 1 h after 30 min of MCAO, without any difference between the groups (G. Andsberg *et al.*, unpublished observations; see also Zhao *et al.*, 1994).

In conclusion, the present results indicate that elevated levels of NGF locally in the striatum increase the resistance of striatal projection neurons to cell death caused by transient focal ischaemia. However, it must be underscored that our data do not show whether biological delivery of NGF might become of therapeutic value in patients with stroke. It now seems highly warranted to clarify the mechanisms of neuroprotection by NGF and when, in relation to the ischaemic insult, the neurotrophic factor has to be delivered to obtain this effect. Of particular importance will be to explore in animal models whether treatment with NGF can also ameliorate stroke-induced behavioural deficits.

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Abbreviations

ChAT	choline acetyltransferase
DARPP-32	dopamine- and adenosine 3′:5′- monophosphate-regulated phosphoprotein with a molecular weight of 32 kilodalton
MCAO	middle cerebral artery occlusion
NeuN	neuronal nuclear protein
NGF	nerve growth factor

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Generation and Transplantation of EGF-Responsive Neural Stem Cells Derived from GFAP–hNGF Transgenic Mice

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EGF-responsive neural stem cells isolated from murine striatum have the capacity to differentiate into both neurons and glia *in vitro*. Genetic modification of these cells is hindered by a number of problems such as gene stability and transfection efficiency. To circumvent these problems we generated transgenic mice in which the human GFAP promoter directs the expression of human NGF. Neural stem cells isolated from the forebrain of these transgenic animals proliferate and form clusters, which appear identical to stem cells generated from control animals. Upon differentiation *in vitro*, the transgenic stem cell-derived astrocytes express and secrete bioactive hNGF. Undifferentiated GFAP–hNGF or control stem cells were transplanted into the striatum of adult rats. One and 3 weeks after transplantation, hNGF was detected immunocytochemically in an halo around the transplant sites. In GFAP–hNGF–grafted animals, intrinsic striatal neurons proximal to the graft appear to have taken up hNGF secreted by the grafted cells. Ipsilateral to implants of GFAP–hNGF-secreting cells, choline acetyltransferase-immunoreactive neurons within the striatum were hypertrophied relative to the contralateral side or control-grafted animals. Further, GFAP–hNGF-grafted rats displayed a robust sprouting of p75 neurotrophin receptor-positive fibers emanating from the underlying basal forebrain. These studies indicate that EGF-responsive stem cells which secrete hNGF under the direction of the GFAP promoter display *in vitro* and *in vivo* properties similar to that seen following other methods of NGF delivery and this source of cells may provide an excellent avenue for delivery of neurotrophins such as NGF to the central nervous system. © 1997 Academic Press

INTRODUCTION

Until recently, it was thought that cells of the central nervous system (CNS) were essentially nonmitotic with a limited capacity for self-renewal. This inability would limit the potential for the CNS to repair after injury. Recently, however, a population of actively dividing cells within the subventricular zone of the adult mouse brain has been identified (32). In addition, Reynolds and Weiss (36) isolated a population of cells from the embryonic and adult mouse striatum which can be expanded indefinitely *in vitro*. In the continued presence of epidermal growth factor (EGF), these cells proliferate and form small clusters of stem cells and stem cell progeny. *In vitro*, these stem cells can differentiate into the three major neural phenotypes (neurons, astrocytes, and oligodendrocytes), following removal of EGF and the addition of a small amount of fetal bovine serum (FBS) (37, 38, 43). Clonal analysis of these cells indicates that a single cell can give rise to a cell cluster containing neurons, astrocytes, and oligodendrocytes following differentiation (37). These data indicate that these cells possess the characteristics of a CNS stem cell including the capacity for self-renewal and multipotentiality.

The identification of stem cells in the CNS offers a new strategy for treatment of neurodegenerative diseases, as these cells may provide an optimal source of donor material for direct transplantation into the diseased or injured CNS (10, 15, 42). Currently, CNS transplantation studies primarily employ primary fetal tissue. The limited availability of suitable tissue, coupled with the ethical, technical, and safety issues surrounding the transplantation of human fetal tissue, poses a significant impediment to clinical progress. The ideal source of tissue for neural transplantation would be cells that can be exponentially expanded in culture without the use of oncogenes, banked, tested for the absence of adventitious agents, and cryopreserved. In addition, these cells should be capable of differentiating into appropriate phenotypes based upon environmental cues. Neural stem cells provide such an option in that they fulfill all of these requirements.

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While transplantation of neural stem cells into the CNS may provide a means for cellular replacement, transplantation of stem cells genetically modified to deliver growth factors would provide several advantages. Endogenous release of growth factors may induce the stem cells toward appropriate neuronal phenotypes which could facilitate cellular replacement. These cells could integrate into the surrounding tissue and secrete growth factors directly into the parenchyma. These integrated cells would provide trophic support to locally damaged cells and could rescue cells from damage or death in disease states. In fact, the secretion of growth factors such as NGF or BDNF from transplanted HiB5 neural cell lines can protect striatal neurons from excitotoxic damage (30). *In vitro,* growth factors such as BDNF (1) have been shown to enhance the differentiation of neuronal phenotypes from stem cells. In addition, hNGF secreted from encapsulated cells will induce hypertrophy and sprouting of the endogenous population of neurons (24) and NGF secreted from fibroblast cell lines will protect against excitotoxic damage such as quinolinic acid lesions (11–14, 39).

To date, genetic modification of neural cells suffers from problems of toxicity, genetic instability, and inadequate transfection or infection efficiency. These inherent problems make the application of genetically modified stem cells for transplantation difficult. However, isolation of neural stem cells from transgenic mice in which promoter elements direct the expression of a gene of interest provides a more stable and efficient method of genetic modification (18). In the work described here, EGF-responsive stem cells have been generated from transgenic animals in which the GFAP promoter directs expression of hNGF. Upon differentiation, these stem cells are induced to form mature astrocytes which secrete bioactive hNGF. We have used these cells for transplantation into the adult rat striatum. These grafts prove to be functional since they secrete NGF *in vivo* which induces the hypertrophy and sprouting of the endogenous cholinergic neurons within the rodent forebrain.

METHODS

GFAP–hNGF Construct

Materials. All enzymes were purchased from Boehringer Mannheim (New York, NY). The cloning vectors pBS-KS(+), pUC18, and pcDNA1/Neo were obtained from Stratagene (La Jolla, CA), Boehringer Mannheim, and Invitrogen (La Jolla, CA), respectively. G418 and Dulbecco's modified Eagle medium (DMEM) were from Gibco (Grand Island, NY). The calcium phosphate transfection kit was purchased from CloneTech (San Diego, CA).

Construction of the pGFAP–DHFR-1–hNGF expression vector. The pGFAP–DHFR-1–hNGF expression vector was generated through a three-step cloning process involving the construction of three intermediary cloning vectors: pBS(DX-B)–GFAP–rI2–MP1, pNUT–MP1–rI2–D*Sal*I–*Pac*I–*Asc*I, and pGFAP–DHFR-1. Briefly, the pBS-KS(+) plasmid was digested by *Bam*HI and *Xho*I, overhanging termini were filled in with DNA polymerase I (Klenow fragment), and the resultant plasmid was self-ligated. The resulting plasmid pBS(DX-B) was digested by *Not*I and ligated to a 2964-bp fragment isolated from pGFAP–rI2–MP1–PA2 that was digested by *Bgl*II, Klenow filled in, and ligated to a *Not*I linker. The resulting plasmid was named as pBS(DX-B)–GFAP–rI2–MP1. The pNUT–MP1–rI2 was *Eco*RI digested and overhanging termini were filled in with DNA polymerase I (Klenow fragment) and ligated to a *Pac*I linker. A 3216-bp *Sal*I/*Bam*HI fragment containing the MP1 and pUC18 regions isolated from the above resulting plasmid pNUT–MP1–rI2–*Pac*I was ligated to a 3083-bp *Sal*I/*Bam*HI fragment containing the DHFR, MT-1, and rI2 regions isolated from pNUT–MP1–rI2–D*Sal*I. The resulting plasmid pNUT–MP1–rI2–D*Sal*I–*Pac*I was then *Sal*I digested and then overhanging termini were filled in with DNA polymerase I (Klenow fragment), ligated to an *Asc*I linker, and self-ligated generating the final intermediary cloning vector pNUT–MP1–rI2–D*Sal*I–*Pac*I–*Asc*I. A 5438-bp *Not*I/*Bam*HI fragment isolated from pNUT–MP1–I2–D*Sal*I–*Pac*I–*Asc*I was ligated to a 2434-bp *Not*I/*Bam*HI fragment isolated from pBS(DX-B)–GFAP–rI2–MP1. The resulting plasmid was named pGFAP–DHFR-1. The pGFAP–DHFR-1–hNGF expression vector was constructed by subcloning a 2508-bp fragment containing the hNGF from pcDNA1/Neo/hNGF into the pGFAP–DHFR-1 intermediary cloning vector (Fig. 1). The resulting plasmid pGFAP–DHFR-1–hNGF was sequenced in order to verify the hNGF insertion orientation.

In order to verify the functionality of the construct, C6 glioma cells (1×10^6) were transfected with 10 μg of pGFAP–DHFR-1–hNGF plasmid and 1 μg of pcDNA1/Neo plasmid using the calcium phosphate kit from CloneTech. Forty-eight hours after transfection, the culture medium was supplemented with 1 mg/ml of G418. Stably transfected cells were maintained in medium containing 1 mg/ml of G418. Bioactive hNGF secretion from these cells was determined by ELISA and PC12 neurite outgrowth.

Transgenic Mouse Production

The 7695-bp DNA fragment containing the GFAP–rI2–hNGF–MP1 and SV40–mDHFR–HBV3′ sequences was generated by *Pac*I and *Asc*I digestion and size selected by agarose electrophoresis (29). DNA was purified by the β-agarose treatment method according to the manufacturer's instructions. Transgenic mice

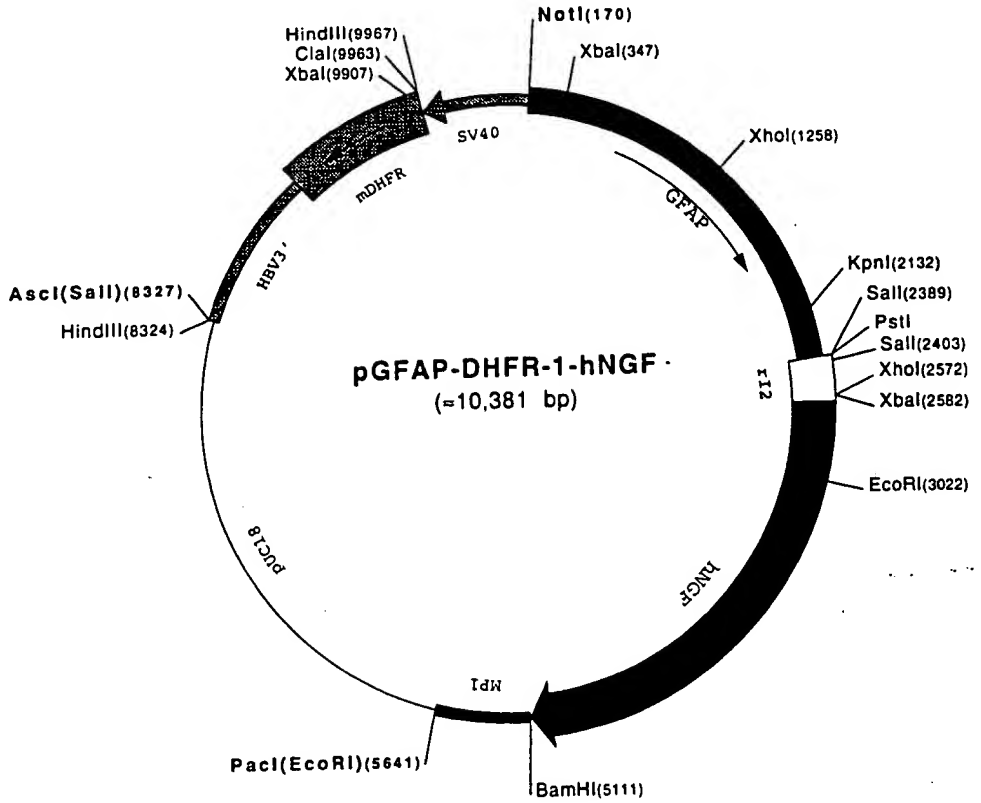

FIG. 1. GFAP–DHFR–hNGF expression vector used for the generation of transgenic mice. The GFAP promoter is fused to the hNGF coding sequence. The mutant DHFR driven by the SV-40 promoter allows for amplification of this construct.

were produced according to standard techniques. Approximately 2 pl of DNA solution was microinjected into the male pronucleus of fertilized eggs obtained from the mating of FVB/N mice. The injected eggs were then transplanted into pseudo-pregnant females. Integration of the transgene into the mouse genome was determined by PCR using unpurified DNA extracts from tissue digests according to the method of Hanley and Merlie (19). After removal of the striata from each fetus the remaining brain tissue was used for the DNA digest. The PCR primers used for transgenic determination were 5′-TGC TCG CGG GGA TCT CTG CAG GTC GGT CGA CAA-3′ and 5′-ATA CAC TGT TGT TAA TGT TCA CCT CTC CCA ACA CCA-5′. Tissue for cell culture was isolated from fetuses as described below.

Stem Cell Isolation and Culture

Stem cells were isolated from the striata of E14.5–E15.5 mice as previously described (36). Briefly, striata were removed from each fetus and mechanically dissociated using a fire-polished pipette. Separate cultures were generated from each fetus. Cell suspensions were grown in N2, a defined DMEM:F12-based (Gibco) medium containing 0.6% glucose, 25 μg/ml insulin, 100 μg/ml transferrin, 20 n*M* progesterone, 60 μ*M* putrescine, 30 n*M* selenium chloride, 2 m*M* glutamine, 3 m*M* sodium bicarbonate, 5 m*M* Hepes. This medium was supplemented with 20 ng/ml murine EGF (Collaborative Research). Typically, the cells grew in clusters which were passaged by mechanical dissociation approximately once each week and reseeded at approximately 60,000–75,000 cells/ml.

Stem cells were differentiated by initially plating on polyornithine-coated glass coverslips. In these experiments, stem cells were plated as clusters or as a single cell suspension. To induce differentiation, EGF was removed from the growth medium and the medium was supplemented with 1% FBS (Gibco). Cells were cultured for 5–10 days before fixation for immunocytochemistry.

Bioassay for hNGF

To assay hNGF output from the individual stem cell cultures, the cells were first differentiated in FBS as described above. Stem cells were plated as clusters on polyornithine-coated plastic culture dishes. The cells were grown in N2 defined medium supplemented with 1% FBS for either 1 or 2 weeks and were fed twice each week. At the designated time points, the conditioned medium (3 day conditioned) was removed and used for the bioassay. The cultures were then placed in PC-1 defined medium (BioWhitaker) for 24 h. At the end of

this pulse the PC-1 medium was removed and the hNGF levels were determined by ELISA. The number of cells in each culture was then determined by trypsinizing the cells and counting them with a hemacytometer.

The conditioned medium was used to induce PC12a cell neurite outgrowth. In these assays, PC12a cells were plated at 25,000 cells/cm^2 in DMEM (Gibco) supplemented with 10% FBS. The next day the medium was removed and replaced with a 1:1 mix of DMEM with 10% FBS and the conditioned medium from the stem cell cultures. The PC12a cells were then evaluated at 24 and 48 h to determine extent of neurite outgrowth. Outgrowth was scored qualitatively and compared to cultures which received recombinant NGF (Boehringer, Mannheim). These data were only used to determine if the secreted NGF was bioactive, rather than to quantify the extent of bioactivity.

Immunocytochemistry

The stem cell cultures were fixed for 10–20 min at room temperature with 4% paraformaldehyde and then washed three times in 0.1 *M* PBS, pH 7.4. The cultures were then permeabilized using a 2-min incubation in 100% EtOH and washed again with 0.1 *M* PBS. Cultures were then incubated in 5% NGS (normal goat serum) in 0.1 *M* PBS with 1% Triton X-100 (Sigma) for 1 h at room temperature. Primary antibodies were diluted in 1% NGS + 1% Triton X-100 for 2 h at room temperature. The cultures were washed in PBS and incubated with secondary antibodies diluted in 1% NGS with 1% Triton X-100 for 30 min at room temperature in the dark.

The primary antibodies used were: Nestin, 1:500 (rabbit polyclonal, generously provided by R. McKay); O_4, 1:25 (monoclonal, Boehringer Mannheim); β-tubulin, 1:1000 (monoclonal, Sigma); and GFAP, 1:500 (polyclonal, DAKO). The secondary antibodies used in these experiments were goat anti-mouse–FITC (1:128) and goat anti-rabbit–TRITC (1:80) (both from Sigma).

NGF ELISA

Quantitation of hNGF released from differentiated GFAP–hNGF cells was performed as described previously (24).

Animal Subjects

Adult male Sprague–Dawley rats (Taconic Breeders, Germantown, NY) approximately 3 months old and weighing 300–350 g were used in these studies. The animals were housed in groups of three to four in a temperature- and humidity-controlled colony room which was maintained on a 12-h light/dark cycle with lights on at 0700 h. Food and water were available ad libitum throughout the experiment. Beginning 1 day before transplantation, all animals received daily ip injections of 1 mg/kg cyclosporin. After 2 weeks, cyclosporin injections were replaced by oral immunosuppression (Neoral, Sandoz). All experimentation was conducted in accord with NIH guidelines.

Surgery

Rats were anesthetized with sodium pentobarbital (45 mg/kg, ip) and positioned in a Kopf stereotaxic instrument. A midline incision was made in the scalp and a hole was drilled for the injection of cells into the striatum. Rats received unilateral implants into the left striatum using a glass capillary attached to a 10-μl Hamilton syringe, at two sites with control stem cells (cell line 74-43, $n = 8$) or GFAP–hNGF stem cells (cell line 74-61, $n = 8$). The stereotaxic coordinates for implantation were: +0.2 mm anterior to Bregma, 3.2 mm lateral to the sagittal suture, and 5.4 mm below the cortical surface; and −0.2 mm posterior to Bregma, 3.2 mm lateral to the sagittal suture, and 5.4 mm below the cortical surface (35). The cells were unilaterally injected in two 1.0-μl deposits at each injection site by injecting one deposit at 5.4 mm and another at 4.9 mm ventral from the cortical surface, and then 1 min of diffusion time was allowed after the first injection and an additional 3 min of diffusion time was allowed after the final injection. Each animal received a total of 250,000–500,000 cells in a total volume of 2 μl. Cells were transplanted 1–2 days after passaging and the cell suspension was made up of undifferentiated stem cell clusters of 5–20 cells. In this experiment stem cells were implanted at passage 34 (control) or passage 35 (GFAP–hNGF). Following implantation, the skin was sutured closed.

Histology

Animals from the initial experiment were transcardially perfused using a peristaltic pump with 20 ml saline followed by 500 ml of 4% paraformaldehyde. All solutions were ice cold (4°C) and prepared in 50 m*M* PBS (pH 7.4). Brains were removed following fixation, placed in 25% buffered sucrose (pH 7.4), and refrigerated for approximately 48 h. Sections throughout the entire striatum were cut at 40-μm intervals on a cryostat and stored in a cryoprotectant solution. Adjacent sections through the striatum were processed for the immunocytochemical localization of choline acetyltransferase (ChAT; 1:1000, Chemicon) as previously described (11, 25) using the labeled antibody procedure (21). One series of sections was stained for glutamic acid decarboxylase (GAD; 1:7500, Oncogene) with nickel intensification using a modification of the above procedure (see 25 for details). Another series of sections was stained using M2, a mouse-specific marker (generously provided by Dr. Carl Lagenaur; 1:20 or 1:50). All immunohistochemical reactions were terminated by three 1-min rinses in PBS. Sections were mounted, dehydrated,

and coverslipped. Control sections were processed in an identical manner except the primary antibody solvent or an irrelevant IgG was substituted for the primary antibody. It is important to note that even though staining was eliminated in sections in which the primary antibody was deleted or an irrelevant IgG was substituted, the potential for antiserum to react with structurally related proteins cannot be excluded. Thus, a degree of caution which is inherent to immunohistochemical procedures is warranted. In this regard, the terms ChAT, GAD, or GFAP immunoreactivity in this study refers to -"like" immunoreactivity (IR). A separate series of sections through the striatum were stained for NADPH–diaphorase (NADPH-d; Sigma) as described previously (2). Adjacent sections were stained for Nissl to aid in cytoarchitechtonic delineation.

Nerve growth factor antibody production and characterization. We used a polyclonal antibody directed against mouse NGF to determine spread of this neurotrophin from grafted cells and to examine the potential uptake of graft-derived hNGF within host striatal neurons. Antibody production and characterization have been reported previously (7, 8, 24, 33).

Nerve growth factor-like immunohistochemistry. Animals were prepared for NGF immunocytochemistry. These animals were perfused transcardially with cold 0.9% saline followed by fixation with 2% paraformaldehyde + 0.2% parabenzoquinone in 0.1 *M* phosphate buffer (pH 7.4; see 7, 8, 33). Histological sections were then processed for NGF-like immunohistochemistry with a rabbit polyclonal antibody against mouse β-NGF according to a modification of our previously described procedure (7, 8, 24, 33). Immunocytochemical controls were included as described previously (24).

Quantitative Morphometric Analysis

Morphometric analysis of cell area was carried out using the NIH Image analysis system. Coronal sections through the striatum at the level of the graft were analyzed. Every ChAT- or GAD-immunoreactive neuron within the striatum of each analyzed section was measured using a computer mouse to manually trace the borders of the immunoreactive perikarya. The size of the cell soma of ChAT or GAD neurons within the striatum of each section analyzed was quantified by an individual blinded to the animal's experimental condition.

RESULTS

GFAP-hNGF Transgenic Animals

For these experiments, transgenic mice were generated by microinjection as described under Methods. The DNA construct consisted of the hGFAP promoter driving expression of the hNGF gene, as shown in Fig. 1. All of the fetuses (transgenic and nontransgenic) were anatomically indistinguishable. Of the 82 fetuses examined, 20 (24%) were positive for the transgene by PCR. Individual EGF-responsive stem cell cultures were generated from the striata from each fetus. Stem cell-derived astrocytes from these fetuses displayed varying levels of hNGF secretion, as described below.

Isolation and Characterization of EGF-Responsive Stem Cells from GFAP–hNGF Transgenic Mice

The striatum was isolated from individual transgenic animals as described under Methods. The striata were dispersed into a single cell suspension and the cells were seeded in EGF-containing medium. Within 5–7 days, the cells proliferated, forming clusters of cells which appeared identical to the EGF-responsive stem cells isolated from nontransgenic controls. After about 1 week *in vitro,* the clusters were mechanically dissociated and the single cells grew into clusters again. Individual stem cell cultures from both transgenic and control animals were passaged about once per week. Analysis of cell number and cell division ensured that the clustering of the cells was the result of proliferation rather than aggregation. Cells isolated from GFAP–hNGF mice or from control littermate animals doubled every 1.2–2.8 days without differences between the proliferation rate of the transgenic and control animals. In addition, stem cell clusters isolated from both transgenic animals and control animals were immunopositive for nestin, an intermediate filament protein (Fig. 2A) in neural stem cells (27).

Neural stem cells from several of the GFAP–hNGF lines were also assessed for their ability to differentiate into all of the major neural phenotypes (neurons, astrocytes, and oligodendrocytes). In these experiments differentiation was induced by culturing the cells on polyornithine, removing EGF, and adding 1% FBS. As demonstrated in Figs. 2B–2D, positive immunoreactivity was found using antibodies which recognize neurons (β-tubulin), astrocytes (GFAP), and oligodendrocytes (O4). These data indicate that the stem cells derived from the transgenic animals are multipotent. Furthermore, this multipotentiality was observed in cells which had been passaged as many as 25 times (Figs. 2B–2D).

The ability of the differentiated cells to express hNGF was also examined. Because hNGF expression is driven by the GFAP promoter, hNGF is predicted to be expressed by the differentiated astrocytes. In these experiments, the individual stem cell cultures were induced to differentiate as described under Methods. At either 1 or 2 weeks postdifferentiation, the conditioned medium from each of the cultures was evaluated for hNGF by ELISA. Expression levels ranged from approximately 0.1 to 2.0 ng/ml/50,000 cells/24 h (Table 1). Furthermore, conditioned medium from the differentiated stem cells induced neurite outgrowth from PC12

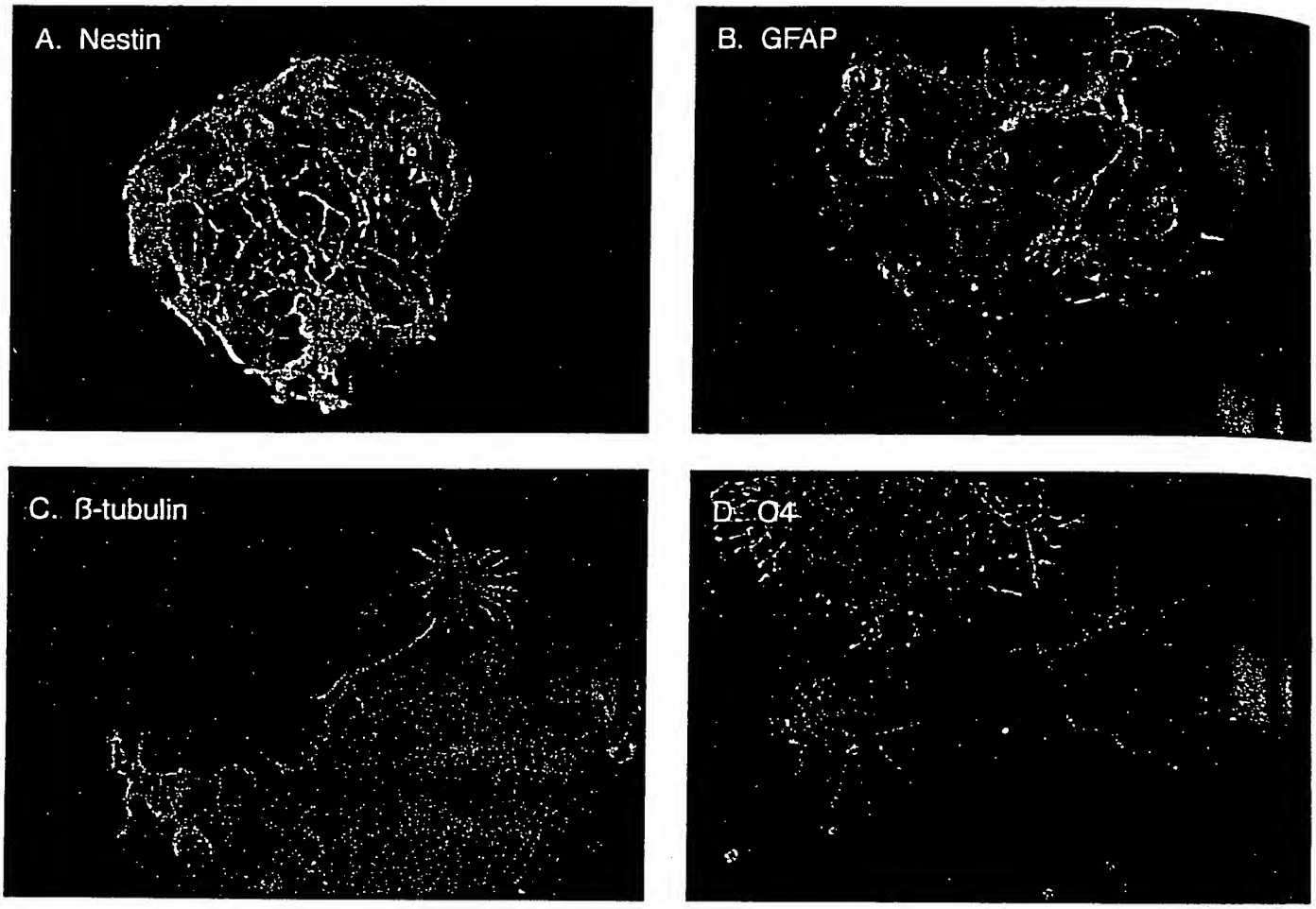

FIG. 2. Immunocytochemical characterization of stem cells derived from transgenic mice expressing GFAP–hNGF. (A) Nestin staining of undifferentiated GFAP–hNGF stem cells. Upon differentiation, these cells generate astrocytes (B), neurons (C), and oligodendrocytes (D). Stem cells were induced to differentiate by culturing them in N2 supplemented with 1% FBS for about 1 week. Note that multipotentiality of these cells is maintained over time, Panels (B–D) are from cells passaged 23–25 times.

cells (Table 1 and Fig. 3), indicating that the hNGF secreted by these cells *in vitro* is bioactive. Human NGF secretion is detectable after passaging the stem cells more than 25 times, suggesting that the cells retain a high level of genetic and phenotypic stability (Table 2). In particular, Table 2 shows the hNGF output from the cell line which was subsequently used for transplantation into rat striatum. With increasing passage, the levels of hNGF from differentiated astrocytes remained high, and up to 2 weeks postdifferentiation similarly high levels of NGF were secreted (in contrast to many viral vectors). Although the levels of hNGF secretion vary somewhat, they do not appear to decline with increasing passage. In fact, the highest values of hNGF secretion were from cells passaged 28 times. Even after cryopreservation, the stem cell-derived astrocytes also secrete hNGF (data not shown), indicating the stability of the transgene.

The hNGF output of undifferentiated stem cells has also been measured (Table 3). In most cultures, the NGF levels are not detectable. However there are a few cell lines which have measurable levels of hNGF secretion. This may result from a subpopulation of cells which are differentiating toward an astrocyte lineage and are expressing GFAP. It is also possible that the expression of NGF from the GFAP–hNGF transgene is seen somewhat earlier than the endogenous GFAP gene.

The constructs used to generate the GFAP–hNGF mice contain the mutant dihydofolate reductase gene (DHFR). We attempted to increase NGF secretion levels in these cells using MTX selection. In these experiments, GFAP–hNGF cell lines 74-46 and 74-61 were selected with either 200 n*M* MTX or 1 μM MTX. Selection continued for at least 2 months before assessing NGF output by the stem-cell-derived astrocytes.

NGF output from cell line 74-46 increased approximately 9- to 16-fold upon selection with 200 n*M* and 1 μM MTX selection (data not shown). However, cell line 74-61 NGF output did not show a significant increase

TABLE 1

NGF Levels in Conditioned Medium from Differentiated GFAP–hNGF Neural Stem Cells

Cell line	Passage	Genomic PCR	NGF/50K cells (pg/24 h) 1 week	PC12 bioassay 1 week	NGF/50K cells (pg/24 h) 2 weeks	PC12 bioassay 2 weeks
74-21	23	–	0	–	0	–
74-43	1	–	0	–	0	–
74-16	11	+	1805	++++	200	++++
74-16	23	+	426	+++	535	+++
74-46	12	+	609	++++	256	++++
74-49	1	+	355	++++	722	++++
74-55	1	+	690	++++	859	++++
74-58	1	+	141	++++	614	++
74-59	1	+	126	++++	301	+
74-61	1	+	2282	++++	2188	+++

Note. Human NGF secretion from stem cells derived from transgenic animals expressing GFAP–hNGF. Neural stem cells were differentiated for 1 or 2 weeks and assayed for NGF output by ELISA. Differentiation of these cells results in astrocyte formation and the activation of the GFAP promoter directing hNGF expression. Values indicate output for 24 h. Conditioned medium from the cultures was also tested on PC12 cells for bioactivity. PC12 cell cultures were ranked for neurite outgrowth from – (no outgrowth) to ++++ (greatest outgrowth).

after MTX selection. Therefore, it seems that one of the cell lines shows an increase in NGF secretion and the other cell line does not show a definite effect. These data suggest that cell lines generated from different transgenic animals may show differences in the effect of selection. Differential effects of MTX on the amplification may reflect the different integration sites of the transgene in each cell line.

Transplantation of GFAP–hNGF and Control Stem Cells

Localization of grafted stem cells using M2 immunohistochemistry. GFAP–hNGF and control cells derived from nontransgenic littermates were transplanted into adult rat striatum. The animals survived either 1 or three weeks posttransplantation. A mouse-specific antibody, which stains primarily glia, but also some neurons (M2), was employed as a species-specific marker for grafted stem cells (28, 34, 46). Using this marker, the grafted cells were successfully identified in all control and GFAP–hNGF-grafted animals (Fig. 4). This antibody predominantly stains the external cell membrane and, as such, perikarya are usually not visualized. In all animals, clusters of M2-IR stem cells were observed, principally within and around the needle tract with M2-positive processes extending for some distance into the surrounding tissue (Fig. 4). Little, if any, migration of M2-IR cells was observed. The exception to this staining pattern was observed principally at the base of injection sites where a dense column of cells within the tract expanded into a teardrop-shaped or round cluster of cells or within the periphery of a graft deposit. At these sites, M2 immunoreactivity was localized within grafted perikarya. Due to the localization of M2 immunoreactivity, quantitation of the number of viable grafted stem cells was not possible. Occasional macrophages were identified in the perigraft area; however, this did not appear to be correlated with the type of cells transplanted.

Expression, secretion, and utilization of graft-derived hNGF. We used a polyclonal antibody raised against hNGF to evaluate the time-dependent expression of hNGF within grafted stem cells, to assess the diffusion of graft-derived hNGF within the host striatum, and to determine whether host striatal neurons could take up graft-derived hNGF. Within the host striatum, hNGF immunoreactivity was not observed in rats receiving implants of control stem cells (Figs. 5A and 6A). In contrast, dense hNGF immunoreactivity was localized within the graft of the GFAP–hNGF cells (Figs. 5B–5E). In addition, a halo of hNGF-IR was observed within the perigraft region in rats receiving implants of hNGF-secreting stem cells (Figs. 6B–6D). This halo of hNGF-IR was not localized to a cellular structure. Rather, the staining pattern was diffuse in nature and localized within the neuropil proximal to the implant site in a manner similar to that seen following implants of hNGF-secreting fibroblasts (26) or NGF infusions (33). The area of hNGF-IR within the host striatum was small and rarely exceeded 2 mm. As was seen within the graft itself, the expression of hNGF-IR within the striatal neuropil decreased with time from transplantation (Fig. 5). Dense hNGF-IR was observed in all rats receiving hNGF-secreting stem cell implants which were sacrificed 1 week posttransplantation (Figs. 6B and 6C). In contrast, hNGF-IR was only discerned in half of the rats receiving identical implants but sacrificed 3 weeks after grafting. In the animals in which hNGF-IR could be seen, the intensity of staining was diminished relative to rats sacrificed only 1 week postgrafting (Fig. 6D).

Within the host striatum of rats receiving hNGF-secreting stem cell grafts, neurons proximal to the implant site exhibited hNGF-IR (Figs. 6C–6F). This staining pattern was absent in rats receiving control stem cell implants. NGF-IR cells were observed within the striatum of rats receiving hNGF-secreting stem cells and sacrificed either at 1 (Figs. 6C and 6E) or at 3 (Figs. 6D and 6F) weeks postgrafting. These cells were small in size (20–30 μm in diameter) and displayed a granular staining pattern within the somata and proximal dendrites. These cells were principally localized within the halo of graft-derived NGF-IR. However, some striatal neurons containing NGF-IR could be seen for short distances outside this halo of secreted NGF.

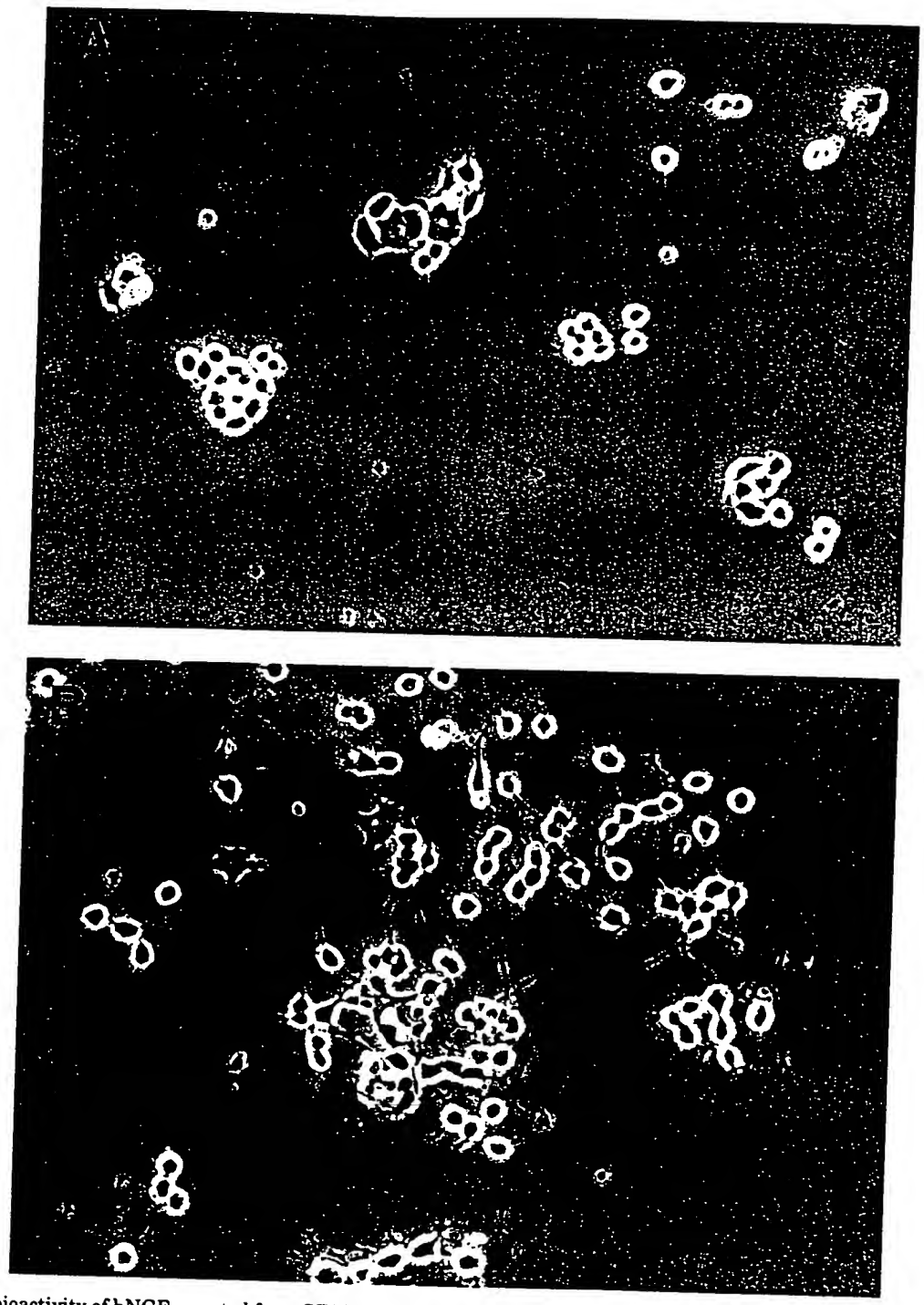

FIG. 3. *In vitro* bioactivity of hNGF secreted from GFAP–hNGF derived astrocytes. Stem cells were differentiated for 2 weeks in medium supplemented with 1% FBS. The conditioned medium from these cultures was placed into PC12a cultures for 48 h. (A) Conditioned medium from control stem cells does not induce neurite outgrowth. (B) Neurite outgrowth is induced by conditioned medium from GFAP–hNGF stem cell progeny.

Effects of hNGF stem cell implants upon the size of striatal neurons. Cellular delivery of NGF has been previously demonstrated to induce hypertrophy and sprouting of cholinergic neurons (23). To determine if the hNGF secreted from the grafted stem cells induced this type of effect, ChAT IR was examined. ChAT-IR neurons were observed bilaterally scattered throughout the striatum; this staining pattern was eliminated following deletion or substitution for the primary antibody. ChAT-IR neurons were rounded and multipolar displaying a morphological profile consistent with identification as cholinergic interneurons (e.g., 23).

The transplant procedure itself did not influence the size of cholinergic striatal interneurons. The size of ChAT-IR striatal neurons ipsilateral to control stem cell implants was similar to that seen on the intact, nongrafted side (Figs. 7A and 7E). In contrast, rats receiving grafts of stem cells genetically modified to secrete hNGF displayed a significant hypertrophy of striatal ChAT-IR perikarya ipsilateral to the implant relative to animals receiving identical grafts of control cells (Figs. 7B and 7F). A different score analysis with regard to cross-sectional area of ChAT-IR neurons revealed that cholinergic striatal neurons were significantly larger in NGF-grafted animals relative to the intact side ($P(t1,3) = 3.17; P < 0.05$) (Fig. 8).

GABAergic neurons were also examined for possible hypertrophy induced by the hNGF. GAD-IR (Figs. 7C and 7D)-positive neurons were observed scattered throughout the striatum bilaterally in all animals in a pattern similar to that seen previously (11, 24). Quantitative evaluation of cell size for GAD-IR-positive cells revealed that these cells were unaffected following implants of hNGF-secreting stem cells (Fig. 8).

Graft-derived sprouting of p75^{NTR}-immunoreactive fibers. Within the host forebrain, p75^{NTR}-IR was localized exclusively within basal forebrain perikarya in a pattern similar to what has been described previously (e.g., 22, 23). Deletion or substitution of the primary antibody resulted in the elimination of specific staining. In every rat receiving implants of hNGF-secreting stem cells, a collection of p75^{NTR}-immunoreactive fibers was observed encompassing the implant site (Figs. 9A–9C). This was true for rats sacrificed both 1 and 3 weeks posttransplantation. In contrast, no such staining pattern was present in any rat receiving control stem cell implants (Fig. 9D). In hNGF-grafted rats, p75^{NTR}-IR fibers surrounded the graft site and continued to traverse dorsally up the needle tract (Fig. 9C). These fibers did not stain for dopamine–β-hydroxylase (data not shown) ruling out the possibility that this innervation could result from ingrowth of sympathetic fibers which are also immunoreactive for p75^{NTR}. Rather, these fibers appeared to emanate from p75^{NTR}-IR cholinergic neurons located within the adjacent basal forebrain. Indeed, processes from cholinergic basal forebrain neurons projected toward and into the perigraft region (Fig. 9A). In rats sacrificed 3 weeks posttransplantation, there was a slight decrease in the density of p75^{NTR}-IR fibers surrounding the graft; however, numerous p75^{NTR}-IR fibers were still evident.

TABLE 2

hNGF Output from GFAP–hNGF-Derived Astrocytes

Cell line	Passage	Assay time point (week)	hNGF (pg/50K cells)
74-61	1	1	2282
74-61	1	2	2188
74-61	12	1	603
74-61	12	2	486
74-61	18	1	2743
74-61	18	2	2045
74-61	18	4	3110
74-61	23	2.5	3331
74-61	28	1	9720
74-61	28	2	6227

Note. Stable secretion of hNGF from a single stem cell line generated from a GFAP–hNGF transgenic mouse. This cell line was passaged 28 times and still demonstrated significant hNGF output. Differentiation of these cells results in astrocyte formation and the activation of the GFAP promoter directing hNGF expression. Values indicate output for 24 h.

TABLE 3

hNGF Levels in Conditioned Medium from Undifferentiated GFAP–hNGF Stem Cells

Cell line	Passage	Genomic PCR	hNGF (pg)	Days of conditioning
74-43	11	–	0	7
74-44	9	–	0	7
74-8	36	+	0	7
74-16	12	+	126	8
74-16	35	+	0	8
74-29	32	+	0	7
74-45	10	+	0	8
74-46	11	+	2320	7
74-48	10	+	0	7
74-49	10	+	0	7
74-55	10	+	0	7
74-58	10	+	0	7
74-59	10	+	0	8
74-61	11	+	4324	7
74-63	10	+	0	7

Note. Human NGF secretion from undifferentiated stem cells derived from transgenic mice in which the GFAP promoter directs expression of hNGF. Neural stem cells were maintained in defined medium containing EGF for the number of days indicated. The conditioned medium was then assayed for hNGF by ELISA.

DISCUSSION

The transplantation of neural stem cells offers an opportunity to protect endogenous cells and facilitate cell repair or replacement. In addition, genetic modification of these cells will facilitate the parenchymal delivery of growth factors. The genetic modification of neural stem cells by traditional methods is hindered by the difficulties in gene stability and transfection efficiency. Furthermore, upon transfection or infection, stem cells may not remain pluripotential or may be fundamentally altered (16). The use of transgenic mice to control expression of the gene of interest avoids these problems. In the present studies we generated transgenic mice in which the GFAP promoter directs the expression of hNGF. The use of the GFAP promoter

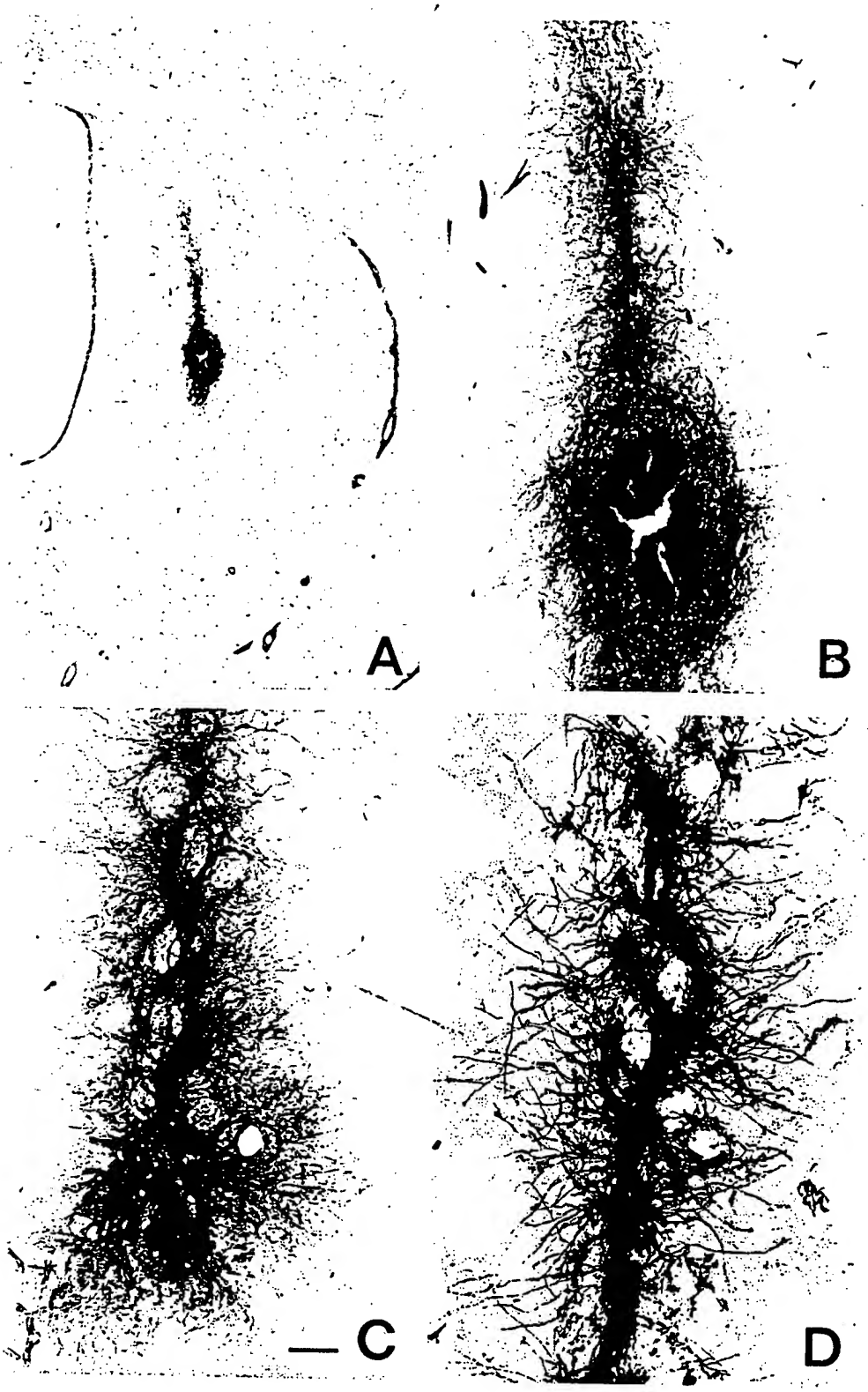

FIG. 4. (A) Low- and (B–D) high-power photomicrographs of M2-immunostained sections illustrating the presence of dense clusters of surviving GFAP–hNGF stem cells 3 weeks after transplantation. Scale bar in C, 500 μm in A; 100 μm in B and C; and 67 μm in D.

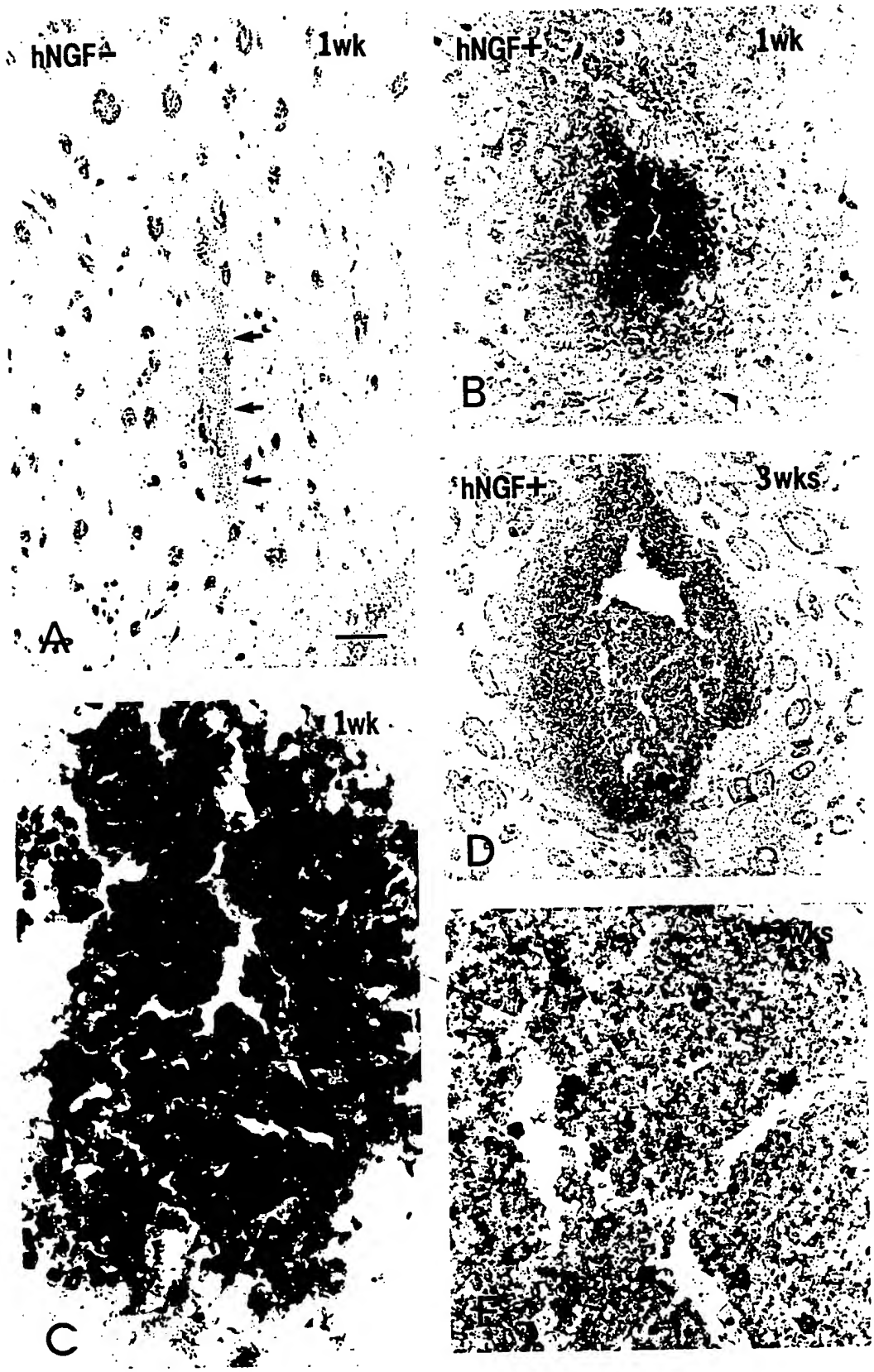

FIG. 5. NGF-immunostained sections through the striatum of stem cell grafted rats. (A) Note the absence of staining in a rat receiving an implant of control (non-NGF secreting) stem cells (arrows). (B) Low-power photomicrograph of a dense cluster of genetically modified grafted stem cells which express NGF immunoreactivity *in vivo* and were sacrificed 1 week posttransplantation. (C) Higher power view of the same cell cluster as in B illustrating that virtually every cell expresses NGF immunoreactivity. (D) Low- and (E) high-power photomicrographs illustrating that fewer grafted stem cells express NGF-IR 3 weeks posttransplantation. Scale bar in A, 250 μm in A; 100 μm in B and D; and 50 μm C and E.

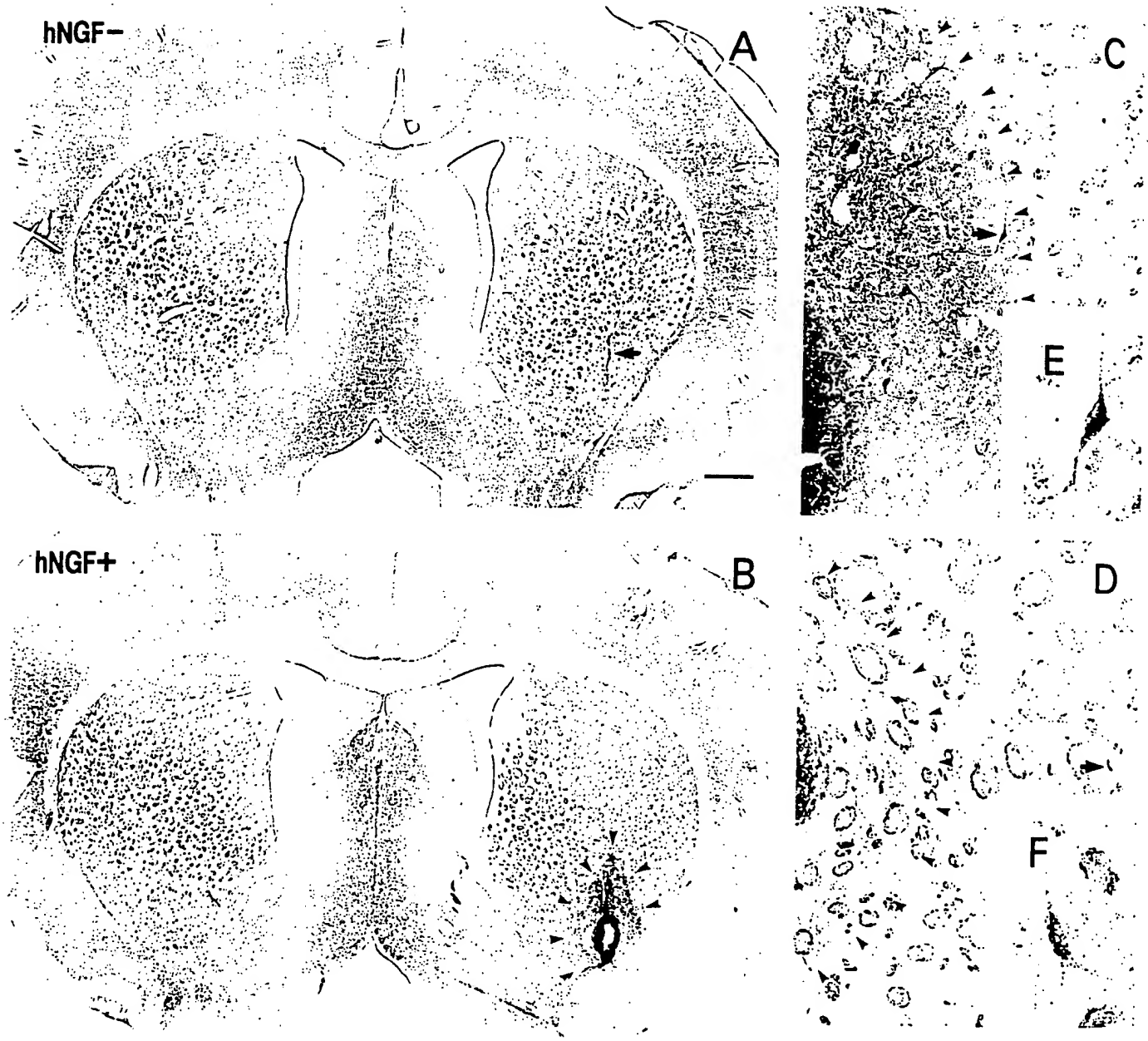

FIG. 6. Low-power photomicrographs of NGF-immunostained sections from rats receiving (A) control or (B) hNGF-secreting stem cell implants and sacrificed 1 week posttransplantation. (A) Note the absence of NGF-IR within and around the graft site (arrow). In contrast, endogenous NGF-IR can be discerned in this animal within the septal/diagonal band complex. (B) In a rat receiving genetically modified stem cells, NGF-IR can be seen within the graft and a halo of graft-derived NGF secreted into the host striatum can be seen (arrowheads). (C) At higher magnification, the halo of graft-derived NGF can be seen (arrowheads). Within the striatum, host striatal neurons appear to have taken up graft-derived hNGF and transported this neurotrophin back to the cell soma. (D) The intensity of NGF-IR within the host was diminished in rats receiving stem cell implants and sacrificed 3 weeks posttransplantation. NGF-IR striatal perikarya could still be observed at this time point. (E and F) High-power photomicrographs of host striatal neurons from rats sacrificed at (E) 1 and (F) 3 weeks posttransplantation. These cells display the characteristic granular staining pattern of neurons which retrogradely transport NGF. Scale bar in A, 1000 μm in A and B; 100 μm in C and D; and 50 μm in E and F.

allows cell-specific expression of the growth factor in astrocytes. Although endogenous mouse GFAP expression is not detected until E16.5 (3, 20), the GFAP–lacZ transgene has been detected as early as E12.5 (4). Therefore, it is possible that hNGF is secreted before harvesting EGF-responsive stem cells at E15. However, endogenous hNGF expression does not appear to interfere with the normal development of the embryos or acquisition of multipotential neural stem cells.

EGF-responsive neural stem cell lines were obtained from a number of founders with chimeric expression of the GFAP–hNGF construct. The stem cells appeared

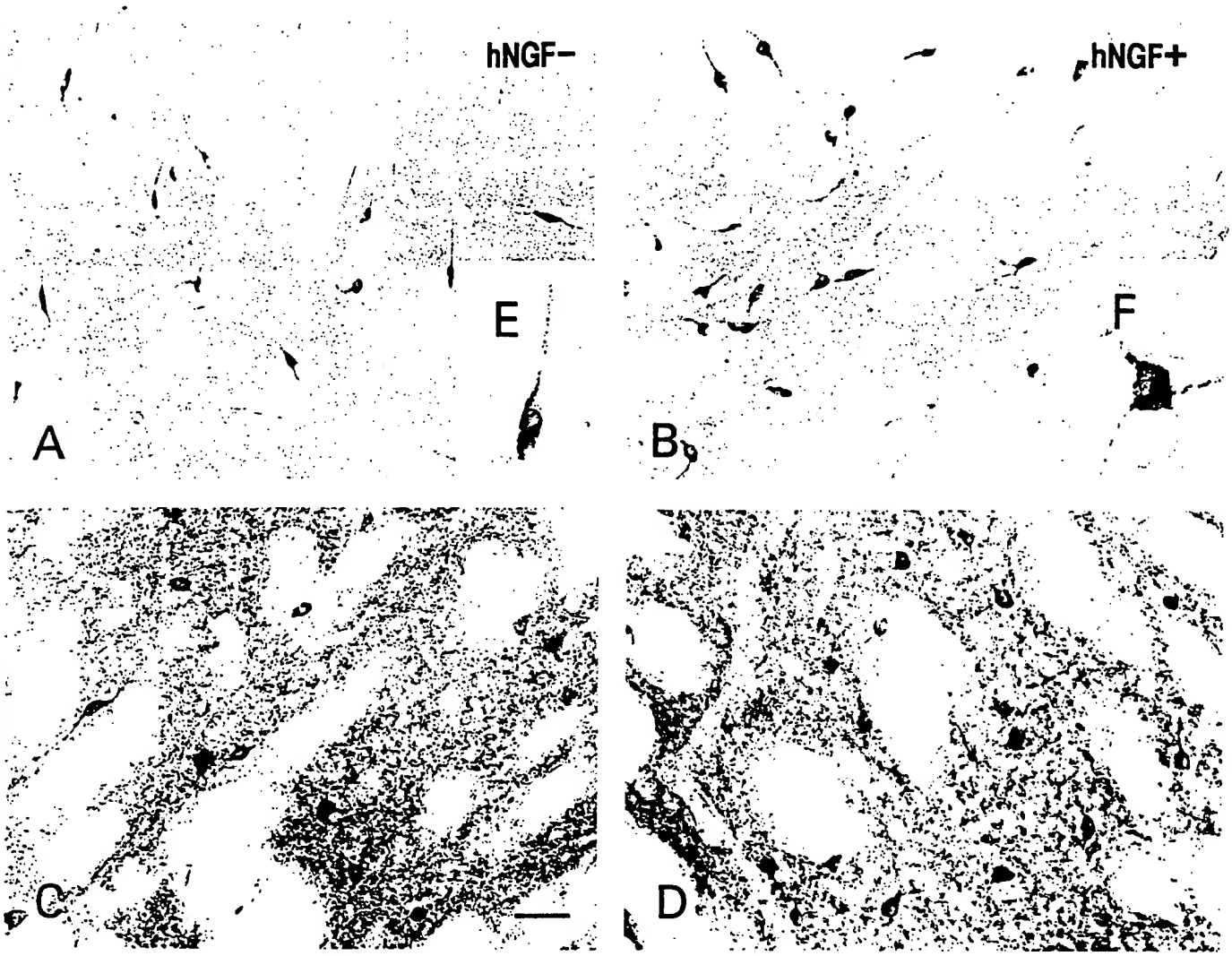

FIG. 7. ChAT (A,B,E,F) and GAD (C,D)-immunostained sections from rats receiving intrastriatal stem cell implants. Note the hypertrophy of ChAT-IR neurons in rats receiving stem cell grafts which were genetically modified to secrete hNGF (B,F) relative to rats receiving control (non-hNGF secreting) stem cell implants (A,E). In contrast, GAD-IR striatal neurons were similar in size in rats receiving control (C) or hNGF (D)-secreting stem cell grafts. Scale bar in C, 250 μm in A–D and 50 μm in E and F.

similar to stem cells generated from littermate control animals as evidenced by their similar proliferation rates and pluripotentiality. Upon differentiation, cell lines generated from different founder animals showed varying levels of hNGF expression. In addition, detectable levels of hNGF were found in conditioned media from some of the undifferentiated GFAP–hNGF stem cell lines. These cultures contain a diversity of cell types, including progenitor cells or astrocyte precursor cells. As the clusters of cells grow in size, the cells which reside in the interior of the cluster are likely to begin differentiating, in part, because they are no longer exposed to the EGF in the medium. It is likely that the hNGF detected in these cultures is the result of a GFAP promoter activity in differentiated astrocytes or astrocyte precursors within the stem cell cultures. Although GFAP is not detected immunocytochemically in the undifferentiated cultures, there may be a low level of GFAP expression which is not detected using immunostaining. This small population of cells may exhibit GFAP expression from the endogenous gene and hNGF expression from the transgene. The NGF detected in these experiments is not due to the secretion of murine NGF by the cultured cells because the ELISA is specific for *human* NGF. Therefore, the levels of hNGF must be the result of transgene expression in this heterogeneous population of cells. *In situ* hybridization studies would allow the determination of which cells in these cultures are expressing hNGF.

As the stem cells were passaged, the multipotentiality of the cells appeared to change. Qualitative observations suggest that the number of neurons formed upon differentiation decreased after about passage 15–20. Although neurons could be identified as late as passage

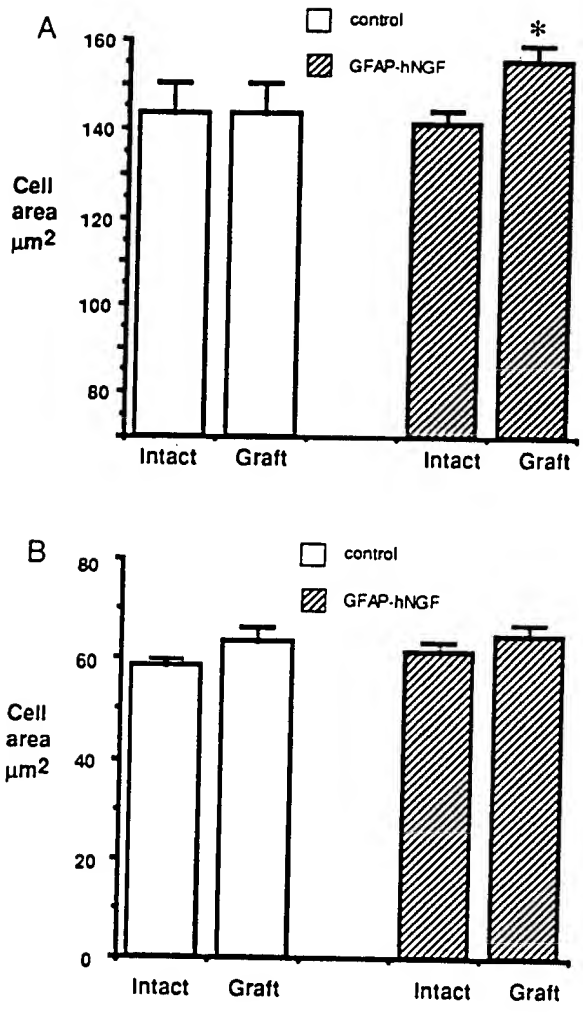

FIG. 8. Quantitation of hypertrophy of cholinergic striatal neurons following implants of GFAP–hNGF or control stem cells. Grafts of GFAP–hNGF cells induced significant hypertrophy in endogenous cholinergic neurons, but not in GABAergic neurons. $^*P < 0.05$.

23 (Fig. 2B), the percentage of neurons within the total population appeared to decrease. This observation was noted in stem cells generated from both control animals and transgenics. Although the percentage of neurons diminished with increasing passage number, the percentage of cells which differentiated into astrocytes appeared similar in control and transgenic animals. In addition, hNGF secretion from the astrocytes derived from transgenic animals did not appear to decrease as the cells were passaged; in fact, the hNGF secretion (hNGF secreted per cell) appears to increase with passage number. Cells were passaged as many as 35 times and, upon differentiation, showed bioactive efficacy. Furthermore, cells which were cryopreserved and subsequently thawed also demonstrated reliable hNGF secretion (unpublished observations) similar to cells which were not previously frozen.

After transplantation into adult rat striatum, both control and transgenic stem cells formed dense grafts which demonstrated significant survival. All of the animals were immunosuppressed throughout the course of the experiment which undoubtedly facilitated the survival of these xenografts. It is not clear if prolonged immunosuppression is necessary for graft survival, although we are currently investigating the effects of different immunosuppression protocols. In addition, using microtransplantation techniques in which there is very little damage to the host probably reduced the host–graft reaction.

Although the stem cells survived well, they did not appear to migrate extensively. This limited migration is likely due to the host environment, rather than to the inability of the cells to migrate. It is recognized that the developing environment is more permissive for the migration of implanted cells (5, 6, 34). Stem cells transplanted into the embryonic (44) or the neonatal (45) brain have shown extensive migration. In the adult mouse brain, the rostral migratory pathway seems to be an exception where migration is permitted for transplanted cells (40). EGF-responsive stem cells labeled with [^{3}H]thymidine before transplantation into the adult striatum showed labeled cells throughout the striatum, but differentiation of these cells was not detected (45), indicating that undifferentiated cells may migrate more efficiently. Furthermore, endogenous stem cells can be induced to proliferate and migrate in the adult mouse by the intraventricular infusion of EGF (9). Therefore, transplanted stem cells appear capable of migration in adult CNS if provided with the appropriate environmental cues. In the experiments described here, the EGF-responsive stem cells have been transplanted into the adult CNS, an environment which is thought to be inhibitory to migration. This finding is consistent with experiments in which rat-derived EGF-responsive stem cells were implanted into 6-OHDA-lesioned rats (adult) and demonstrated minimal migration (41).

In vitro, EGF-responsive stem cells can differentiate into neurons, astrocytes, and oligodendrocytes. However, transplantation of these cells into the adult rat CNS results in the formation of astrocytes (45). In the experiments presented here, immunocytochemistry for the mouse-specific glial marker M2 was used to determine the extent of the graft. Although immunocytochemistry with M2 precludes identification of these cells as oligodendrocytes or astrocytes, it is most likely that the majority of the cells in these experiments have differentiated into astrocytes based on the expression of the hNGF transgene from the astrocyte-specific GFAP promoter.

Upon differentiation *in vitro,* the GFAP–hNGF-derived astrocytes secreted bioactive hNGF as evidenced by the induction of neurite outgrowth in PC12 cells. In addition, a halo of strong NGF immunoreactivity was seen at the implant site 1 week posttransplanta-

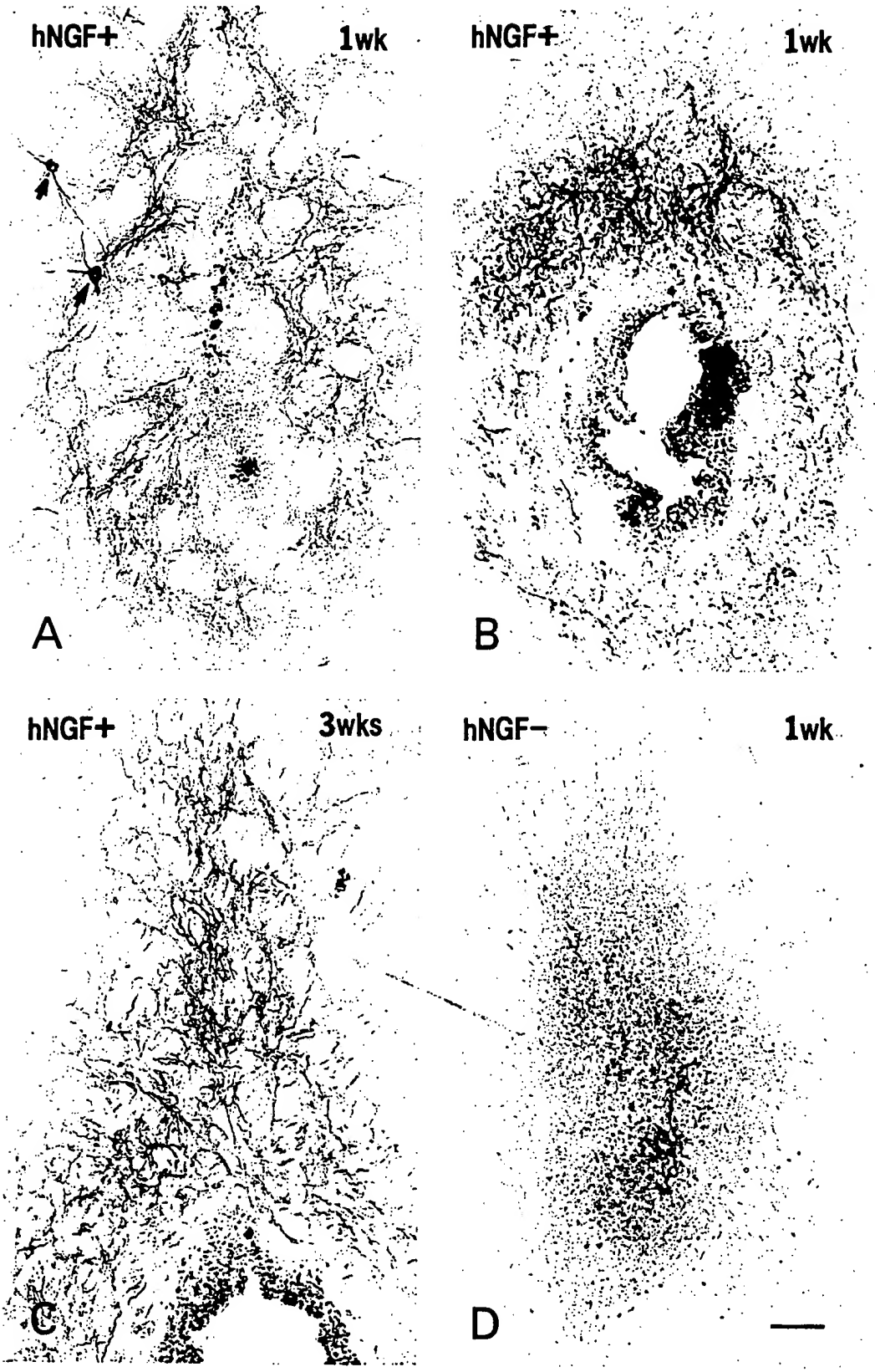

FIG. 9. p75^{NTR}-immunostained sections through the striatum of rats receiving hNGF-secreting (A–C) or control (D) stem cell implants. (A and B) A fine network of p75^{NTR}-IR fibers surround the hNGF-secreting stem cell implants 1 week following implantation. Note the presence of two p75^{NTR}-IR interstitial neurons within the nucleus basalis proximal to the graft site (arrows). (C) This p75^{NTR}-IR fiber staining pattern was still evident 3 weeks posttransplantation. (D) In contrast, no p75^{NTR}-IR fibers were seen proximal to a control stem cell graft. Scale bar in D, 100 μm in A–D.

tion, which decreased somewhat by 3 weeks posttransplantation. M2 staining at both time points appeared similar, indicating that this reduction in staining is not due to cell death within the grafts. It is possible that GFAP promoter activity is increased by gliosis induced by transplantation procedures and that GFAP activity is gradually reduced after the initial gliotic reaction to the implant directly resulting in a decrease in NGF secretion. Longer term studies are in progress to address the continued expression of the NGF once implanted *in vivo*. It is interesting to note, however, that the expression of hNGF in cells which have been differentiated *in vitro* is maintained for as long as 6 months (M. Carpenter, unpublished observations).

Grafts of EGF-responsive cells induced a series of well-characterized NGF effects including hypertrophy of cholinergic striatal neurons, the uptake of graft-derived hNGF by host perikarya, and the sprouting of fibers emanating from the cholinergic basal forebrain. It is likely that these effects are mediated through the activation of the trkA receptor which is located within the cholinergic striatal and basal forebrain cells. Using a polyclonal antibody raised against NGF, we observed NGF-IR at both 1 and 3 weeks posttransplantation. This antibody only visualizes relatively high titres of NGF *in vivo* (see 23 for discussion) and it is likely that the current level of NGF-IR seen in the present study is an underestimate of the actual degree of expression. Indeed, although NGF-IR was diminished at the later time point, a number of lines of evidence suggest that bioactive levels of hNGF were still being secreted by grafted cells at the time of sacrifice. First, the hypertrophy of cholinergic perikarya persisted for up to 3 weeks posttransplantation. We have previously demonstrated that NGF-mediated hypertrophy of cholinergic neurons in intact rats is dependent upon the continued bioavailability of NGF (23). After NGF removal, the hypertrophy of cholinergic striatal neurons dissipates. Thus, the continued hypertrophy of ChAT-IR neurons in the present study indicates that the continued expression of the graft-derived hNGF. Second, the classic NGF sprouting response seen in $p75^{NTR}$-IR basal forebrain neurons was generally equivalent in rats sacrificed 1 or 3 weeks posttransplantation. These findings support the concept that hNGF expression was sustained in these animals for the duration of the experiment.

The use of neural stem cells for transplantation into the CNS offers a number of advantages over transplantation of primary tissue or other cell lines. Primary fetal tissue transplantation relies on the availability of tissue, requiring as many as eight fetuses for a single transplantation into Parkinson's patients (26). In addition, this tissue is only minimally safety tested, resulting in "at risk" transplantation. The use of immortalized cell lines, such as fibroblast cell lines, introduces the risk of tumor formation at the graft site. The use of a stem cell line which will divide continuously *in vitro* and will terminally differentiate into a nonmitotic cell for this type of transplantation resolves both of these issues. Upon transplantation, these cells can differentiate into all of the major neural phenotypes that appear upon differentiation *in vitro*. EGF-responsive stem cells will form oligodendrocytes in the *md* rat and *sh* pup dysmyelinating spinal cord (17, 31). These cells will also differentiate into astrocytes in the adult brain and neurons and astrocytes in the neonatal striatum (45).

The stable genetic modification of EGF-responsive stem cells allows the transplantation of a multipotential cell line that can secrete growth factors which will direct differentiation of the cells. It is possible to engineer stem cells to produce various growth factors or mitogens under the control of cell-specific promoters. The data presented here indicate that EGF-responsive stem cells can be isolated from GFAP–hNGF mice and will remain in an undifferentiated phenotype until induced to differentiate. Upon differentiation, either *in vitro* or *in vivo*, astrocytes derived from these cells will produce bioactive hNGF. The genetic modification of these cells can be extended to include other growth factors. In addition, different cell lines can be combined to deliver multiple factors which have synergistic or complementary effects. This type of system may allow parenchymal delivery of growth factors, and if the cells are induced to migrate (perhaps by coexpression of a factor that will keep the cells in a migratory state), this may allow widespread parenchymal delivery.

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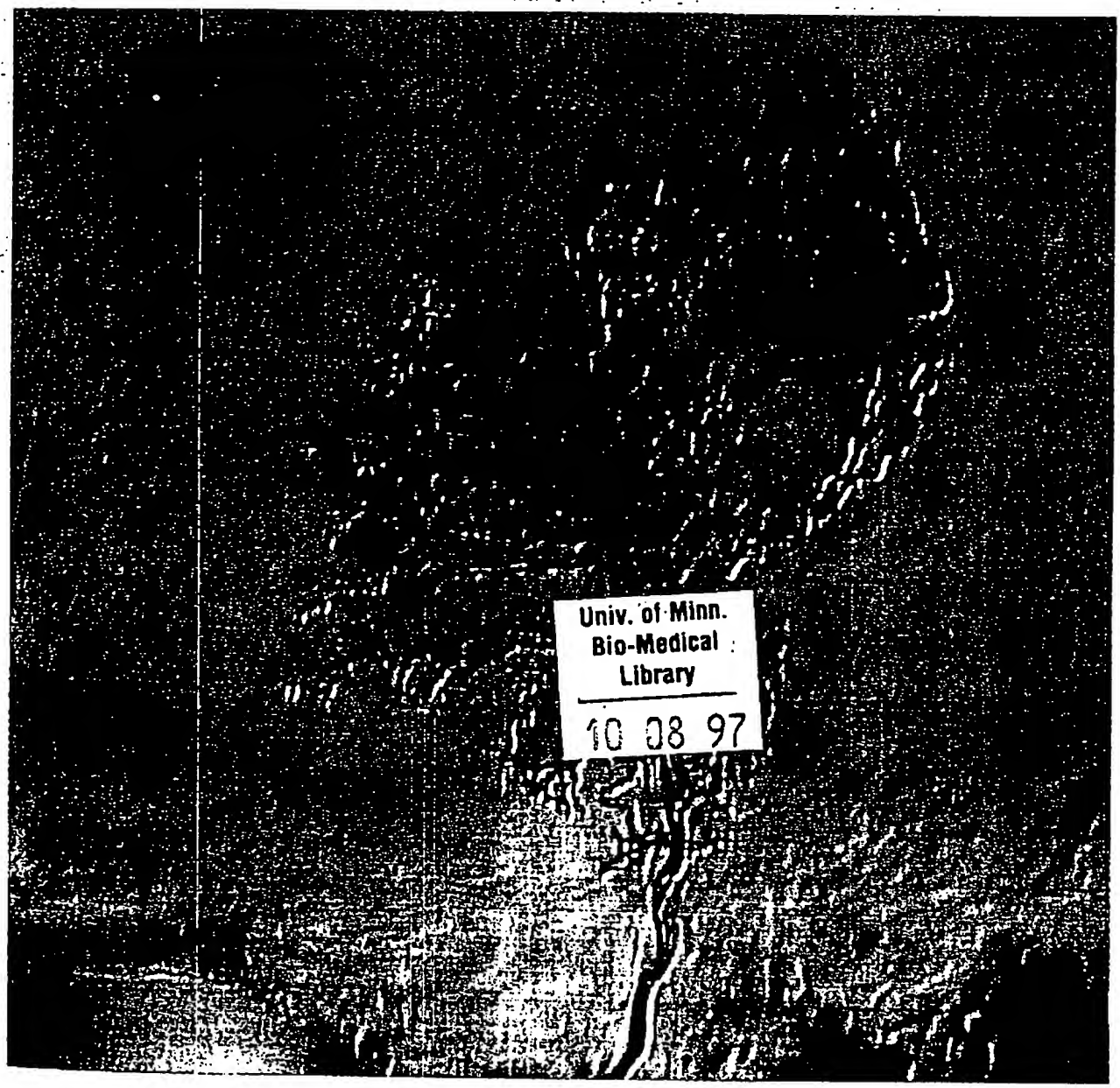

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Grafts of EGF-Responsive Neural Stem Cells Derived From GFAP-hNGF Transgenic Mice: Trophic and Tropic Effects in a Rodent Model of Huntington's Disease

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ABSTRACT

The present study examined whether implants of epidermal growth factor (EGF)-responsive stems cells derived from transgenic mice in which the glial fibrillary acid protein (GFAP) promoter directs the expression of human nerve growth factor (hNGF) could prevent the degeneration of striatal neurons in a rodent model of Huntington's disease (HD). Rats received intrastriatal transplants of GFAP-hNGF stem cells or control stem cells followed 9 days later by an intrastriatal injection of quinolinic acid (QA). Nissl stains revealed large striatal lesions in rats receiving control grafts, which, on average, encompassed 12.78 mm^3. The size of the lesion was significantly reduced (1.92 mm^3) in rats receiving lesions and GFAP-hNGF transplants. Rats receiving QA lesions and GFAP-hNGF-secreting grafts stem cell grafts displayed a sparing of striatal neurons immunoreactive (ir) for glutamic acid decarboxylase, choline acetyltransferase, and neurons histochemically positive for nicotinamide adenosine diphosphate. Intrastriatal GFAP-hNGF-secreting implants also induced a robust sprouting of cholinergic fibers from subjacent basal forebrain neurons. The lesioned striatum in control-grafted animals displayed numerous p75 neurotrophin-ir ($p75^{NTR}$) astrocytes, which enveloped host vasculature. In rats receiving GFAP-hNGF-secreting stem cell grafts, the astroglial staining pattern was absent. By using a mouse-specific probe, stem cells were identified in all animals. These data indicate that cellular delivery of hNGF by genetic modification of stem cells can prevent the degeneration of vulnerable striatal neural populations, including those destined to die in a rodent model of HD, and supports the emerging concept that this technology may be a valuable therapeutic strategy for patients suffering from this disease. J. Comp. Neurol. 387:96–113, 1997. © 1997 Wiley-Liss, Inc.

Indexing terms: striatum; choline acetyltransferase; GABA; NADPH-diaphorase; immunohistochemistry

Huntington's disease (HD) is an inherited neurodegenerative disease characterized by a relentlessly progressive movement disorder with psychiatric and cognitive deterioration. In 1993, it was discovered that an unstable expansion of a CAG trinucleotide repeat in the IT15 gene (The

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Huntington's Disease Collaborative Research Group, 1993) located near the telomere of the short arm of chromosome 4 (Gusella et al., 1983) produces this disease. HD is invariably fatal with an average of 17 years of symptomatic illness. There are no effective treatments (e.g., Martin and Gusella, 1986). Although medications may reduce the severity of chorea or diminish behavioral symptoms, they do not increase survival or substantially improve quality of life as it relates to cognitive state, gait disorder, or dysphagia.

The behavioral sequelae in HD results from a selective vulnerability of striatal neurons. (Ferrante et al., 1985, 1987; Vonsatell et al., 1985; Kowall et al., 1987). Initially, the disease affects gamma-aminobutyric acid (GABA)-containing, medium-sized spiny neurons (Bird and Iverson, 1974; Bird, 1980; Kowall et al., 1987), which innervate the globus pallidus and substantia nigra pars reticulata; critical parts of basal ganglia loop circuitry. Neurochemically, there is a substantial loss of GABA within the striatum (Bird and Iverson, 1974; Bird, 1980; Graveland et al., 1995; Kowall et al., 1987). Large, aspiny interneurons and medium, aspiny projection neurons are less affected and degenerate later in the disease process (Ferrante et al., 1985, 1987; Kowall et al., 1987). Levels of choline acetyltransferase (ChAT), substance P, cholecystokinin, and angiotensin-converting enzyme are decreased (see review Greenamyer and Shoulson, 1994), whereas the expression of some neuropeptides, such as somatostatin and neuropeptide Y, are increased in HD (Beal et al., 1988). In contrast, neurons that contain nicotinamide adenosine diphosphate-d (NADPH-diaphorase), neurotensin, and vasoactive intestinal polypeptide are relatively unaffected (Kowall et al., 1987). Other subcortical and cortical brain regions are involved, but within these extrastriate regions, the degree of degeneration varies; it does not correlate with the severity of the disease and is dwarfed by the striatal changes (see review Greenamyer and Shoulson, 1994).

Currently, therapy for HD is limited and does not favorably influence the progression of the disease. Thus, it is imperative that novel therapeutic strategies be established. In this regard, the grafting of trophic factor-secreting cells has proven successful in experimental models of HD. Our group and others have shown that grafts of nerve growth factor (NGF)-secreting fibroblasts prevent degeneration of striatal neurons destined to die from excitotoxic insult or mitochondrial dysfunction (Schumacher et al., 1991; Frim et al., 1993a,b,c, 1994; Emerich, 1994). Recently, Martinez-Serrano and Björklund (1996) showed that intrastriatal implants of immortalized central nervous system (CNS)-derived progenitor cells that had been genetically modified to secrete NGF also can prevent the neural degeneration in a rodent model of HD.

If transplantation of genetically modified, trophic factor-secreting cells is to be successful clinically for the treatment of neurodegenerative disease, then the optimal means of trophic factor delivery needs to be established. Recently, efforts have been concentrated on developing neural stem cells for transplantation and delivery of biologically active gene products, including trophic factors. There are several advantages to using modified neural stem cells for transplantation. From a practical point of view, neural stem cells can be genetically modified to obtain a relatively homogeneous expression of a desired gene product. This makes them exquisitely suitable for gene transfer to the brain. Neural stem cells have also been shown to survive for long periods of time in vivo and to integrate well within the host tissue without being tumorigenic (Gage et al., 1995; Brustle and McKay, 1996). The present study uses a novel epidermal growth factor (EGF)-responsive stem cell line, which upon differentiation into astrocytes, will secrete human NGF (hNGF) to protect the vulnerable striatal neuronal population following excitotoxic lesions. These stem cells are of particular interest because they are derived from transgenic mice in which the glial fibrillary acidic protein (GFAP) promoter directs the expression of the transgene in vivo. Stem cells generated in this fashion can potentially obviate the problems of toxicity and transient gene expression, which plague other methods of cellular gene therapy. These studies further established that the sparing of striatal cells is associated with the inhibition of vascular permeability and the glial responses observed specifically when tissue sections were probed for the low affinity p75 neurotrophin receptor ($p75^{NTR}$). Lastly, this study shows that hNGF-secreting, stem cell grafts also induce a sprouting response from cholinergic projection neurons located within the basal forebrain.

MATERIALS AND METHODS

Animal subjects

Adult male Sprague-Dawley rats (Taconic Breeders, Germantown, NY) approximately 3 months old and weighing 300–350 g were used in these studies. All experimentation was conducted in accord with NIH guidelines and with the approval of the animal care committee at our institutions. The animals were housed in groups of three to four in a temperature- and humidity-controlled colony room that was maintained on a 12-hour light-dark cycle with lights on at 0700 hours. Food and water were available ad libitum throughout the experiment. All animals received daily injections of cyclosporin (10 mg/kg i.p.), beginning 1 day prior to cell grafting and continuing until the conclusion of the experiment 3 weeks later.

Surgery

Immediately prior to surgery, rats were anesthetized with sodium pentobarbital (45 mg/kg i.p.) and positioned in a Kopf stereotaxic instrument. A midline incision was made in the scalp and a hole drilled for the injection of cells into the striatum. Rats received unilateral implants into the striatum at two sites with cells that were (n = 8) or were not (n = 7) genetically modified to secrete hNGF. The stereotaxic coordinates for implantation were +0.2 mm anterior to Bregma, 3.2 mm lateral to the sagittal suture; −0.2 mm posterior to Bregma, 3.2 mm lateral to the sagittal suture (Paxinos and Watson, 1986). At both sites, the cells were injected in two 0.5-μl deposits with one deposit made at 5.4 mm, and the second at 4.9 mm, ventral to the cortical surface. The cells were injected in the form of small spheres (5–20 cells) by using a glass capillary (O.D. 50–70 μm) connected to a 10-μl Hamilton syringe. Each animal received approximately 500,000 cells at each of the two injection sites. Animals received injections of undifferentiated EGF-responsive stem cells derived from transgenic animals or littermate controls. Following implantation, the skin was sutured closed.

Nine days following grafting, all animals were anesthetized, placed in the stereotaxic instrument, and injected with 225 nmol of quinolinic acid (QA; Sigma Chemical Co., St. Louis, MO) into the previously grafted striatum at the

following coordinates: 1.2 mm anterior to Bregma, 2.6 mm lateral to the sagittal suture, and 5.5 mm ventral to the surface of the brain (Paxinos and Watson, 1986). QA was infused into the striatum through a 28-gauge Hamilton syringe in a 1-μl volume over 5 minutes. The injection cannula was left in place for an additional 2 minutes to allow the QA to diffuse from the needle tip. The skin was then resutured. An additional six rats received QA lesions but were ungrafted and served as additional controls for studies examining the vascular and cellular response to the QA lesion and its prevention by hNGF-secreting stem cell implants. Immediately following the lesion, animals were injected i.p. with 10 ml of a lactated Ringer's solution. Animals were returned to their home cage postoperatively and fed food mash and water ad libitum.

Construction of the pGFAP-DHFR-1-hNGF expression vector

The pGFAP-DHFR-1-hNGF expression vector was generated through a three-step cloning process involving the construction of three intermediary cloning vectors: pBS(DX-B)-GFAP-rI2-MP1, pNUT-MP1-rI2-DSalI-PacI-AscI, and pGFAP-DHFR-1 as described previously (Carpenter et al., 1996; unpublished data). To generate the pBS(ΔX-B)-GFAP-rI2-MP1 intermediary cloning vector, the pBS-KS(−) plasmid was digested by BamHI and XhoI, Klenow filled in, and self-ligated. The resulting plasmid pBS(ΔX-B) was digested by NotI and ligated to a 2,964-bp fragment isolated from pGFAP-rI2-MP1-PA2 that was digested by BglII, Klenow filled in, and added with a NotI-linker. The resulting plasmid was named as pBS(ΔX-B)-GFAP-rI2-MP1. To generate the pNUT-MP1-rI2-DSalI-PacI-AscI intermediary cloning vector, the pNUT-MP1-rI2 (Emerich et al., 1996) was EcoRI digested, Klenow filled in, and added with a PacI linker. A 3,216-bp SalI/BamHI fragment containing the MP1 and pUC18 regions isolated from the above resulting plasmid pNUT-MP1-rI2-PacI was ligated to a 3,083-bp SalI/BamHI fragment containing the dihydrofolate reductase (DHFR), MT-1, and rI2 regions isolated from pNUT-MP1-rI2-DSalI. The resulting plasmid pNUT-MP1-rI2-DSalI-PacI was then SalI digested, Klenow filled in, added with an AscI liner, and self-ligated, generating the final intermediary cloning vector pNUT-MP1-rI2-DSalI-PacI-AscI. To generate the third intermediary cloning vector pGFAP-DHFR-1, a 5,438-bp NotI/BamHI fragment isolated from pNUT-MP1-I2-DSalI-PacI-AscI was ligated to a 2,434-bp NotI/BamHI fragment isolated from pBS(DX-B)-GFAP-rI2-MP1. The resulting plasmid was named as pGFAP-DHFR-1. The pGFAP-DHFR-1-hNGF expression vector was constructed by subcloning a 2,508-bp fragment containing the hNGF from pcDNA1/Neo/hNGF into the pGFAP-DHFR-1 intermediary cloning vector. The resulting plasmid pGFAP-DHFR-1-hNGF was sequenced to verify the hNGF insertion orientation.

Transgenic mouse production

The 7,695-bp DNA fragment containing the GFAP-rI2-hNGF-MP1 and SV40-mDHFR-HBV3' sequences was generated by PacI and AscI digestion and size-selected by agarose electrophoresis (Maniatis, 1982). DNA was purified by the β-agarase treatment method. Transgenic mice were produced according to standard techniques. Approximately 2 pl of DNA solution were microinjected into the male pronucleus of fertilized eggs obtained from the mating of FVB/N mice. The injected eggs were then transplanted into pseudopregnant females. Identification of the integration of the transgene into the mouse genome was performed by PCR by using unpurified DNA extracts from tissue digests according to the method of Hanley and Merlie (1991). Tissue for cell culture was isolated from fetuses as described below.

Stem cell isolation and culture

Stem cells were isolated from the striata of embryonic day (E)14.5–E15.5 mice as previously described (Reynolds et al., 1992). Briefly, striata were removed from each fetus and mechanically dissociated by using a fire-polished pipette. Separate cultures were generated for each fetus. Cell suspensions were grown in N2, a defined DMEM:F12-based (GIBCO, Grand Island, NY) medium containing 0.6% glucose, 25 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 60 μM putrescine, 30 nM selenium chloride, 2 mM glutamine, 3 mM sodium bicarbonate, and 5 mM HEPES. This medium was supplemented with 20 ng/ml murine epidermal growth factor (EGF; Collaborative Research, Bedford, MA). Typically, the cells grew in clusters that were passaged by mechanical dissociation approximately once each week and reseeded at approximately 60,000–75,000 cells/ml.

Stem cells were differentiated by plating on polyornithine-coated glass coverslips. In these experiments, stem cells were plated as clusters or as a single-cell suspension. To induce differentiation, EGF was removed from the growth medium, and the medium was supplemented with 1% fetal bovine serum (FBS).

Bioassay for hNGF

To assay hNGF output and ensure the secretion of hNGF from genetically modified stem cell-derived astrocytes, the stem cells were first differentiated as described above. Stem cells were plated as clusters on polyornithine-coated plastic culture dishes. The cells were grown in N2 defined medium supplemented with 1% FBS for either 1 or 2 weeks and were fed twice each week. At the designated time points the conditioned medium (CM; 3-day conditioned) was removed and used for the bioassay as described below. The cultures were then placed in PC-1 defined medium (BioWhittaker, Walkersville, MD) for 24 hours. At the end of this pulse the PC-1 medium was removed, and the hNGF levels were determined by enzyme-linked immunosorbent assay (ELISA, described below). The number of cells in each culture was then determined by trypsinizing the cells and counting them with a hemacytometer.

The CM was used to induce PC-12a cell neurite outgrowth. In these assays, PC-12a cells were plated in DMEM (GIBCO) supplemented with 10% FBS. The next day the medium was replaced with a 1:1 mix of DMEM with 10% FBS and the conditioned medium from the stem cell cultures. The PC-12a cells were then evaluated at 24 and 48 hours to determine the extent of neurite outgrowth. Outgrowth was scored qualitatively on the basis of outgrowth from cultures that received recombinant hNGF.

NGF ELISA

Quantitation of hNGF released from stem cells was performed on aliquots of CM as follows. Nunc-Immuno Maxisorp ELISA plates were coated with 150 μl per well of anti-mouse-β (2.5S) NGF at 1 ng/ml in coating buffer 1 ×

with phosphate-buffered saline [PBS] without $CaCl_2$ or $MgCl_2$/0.1% sodium azide; pH 9.6). The coated plates were incubated at 37°C for at least 2 hours or alternatively at 4°C overnight. The coating solution was withdrawn from the wells, and the wells were washed three times with 300 µl wash buffer (50 mM Tris-HCl/200 mM $NaCl_2$/1% Triton X-100/0.1% sodium azide; pH 7.0). The wells were then blocked with 300 µl of coating solution containing 10 mg/ml of BSA at room temperature for 30 minutes followed by further washes (three times with 300 µl wash buffer). CM samples were diluted 1:1 two times in sample buffer (the sample buffer is the same as wash buffer, only with 2% BSA), with 10 µl of the prepared samples loaded into the wells. The plates were covered and incubated for at least 2 hours at 37°C or overnight at 4°C. The solutions were removed from the wells by suction and washed three times with 300 µl of wash buffer. To each well, 100 µl of 4 U/ml of anti-mouse-β (2.5S) NGF-β-galactosidase conjugate was added. The plates were incubated at 37°C for at least 1 hour. The solutions were removed from the wells by suction and washed three times with 300 µl of wash buffer. Finally, 200 µl of chlorophenol red-β-D-galactopyranoside substrate solution (40 mg CPRG in 100 mM HEPES/150 mM NaCl/2 mM $MgCl_2$/0.1% sodium azide/1% BSA; pH 7.0) was added to the wells, incubated at 37°C for 30 minutes to 1 hour or after the color development was sufficient for photometric determination at 570 nm, and the samples were analyzed on a plate reader and measured against recombinant NGF protein standards.

Preparation of tissues

Two weeks following the lesion, rats were anesthetized as described above. Prior to perfusion, animals received an injection of heparin into the left ventricle of the heart. Each animal was then perfused transcardially with 0.9% saline, followed by fixation with a 4% paraformaldehyde solution. Brains were then removed from the calvaria, cryoprotected in 30% sucrose in 0.1 M phosphate buffer at 4°C, cut frozen at 40 µm thickness on a sliding knife microtome, and stored at −20°C in cryoprotectant.

Immunocytochemistry

Sections were processed for the immunohistochemical visualization of ChAT, glutamic acid decarboxylase (GAD), $p75^{NTR}$, dopamine β-hydroxylase (DβH), trkA, and M2 (mouse neurofilament antibody specific for the mouse-derived stem cells) via the biotin-labeled antibody procedure (Hsu et al., 1981). For each stain, sections from hNGF and control-grafted animals were processed simultaneously. Residual endogenous peroxidase-containing elements were first removed with a 20-minute incubation in 0.1 M sodium periodate in Tris-buffered saline. Background staining was then blocked with a 1-hour incubation in a Tris-buffered saline solution containing 3% normal serum, 2% bovine serum albumin, and 0.05% Triton X-100. The sections were then incubated in the primary ChAT (1:7,500; Chemicon, Temecula, CA); GAD (1:20,000; Chemicon), $p75^{NTR}$ (1:7,500; Oncogene, Uniondale, MA), DβH (1:2,000, Eugene Tech., Allendale, NJ), trkA (1:10,000; kindly provided by Dr. Louis Reichardt), or M2 (generously provided by Dr. Carl Lagenaur; 1:20 or 1:50) antibody for 48 hours. Sections were then incubated for 1 hour in the appropriate biotinylated IgG antibody (Vector, Burlingame, CA; 1:200). Following washes, sections were placed in the avidin-biotin (ABC "Elite" [Vector]) substrate (1:500) for 75 minutes. Sections were then reacted in a chromagen solution containing Tris-buffered saline, 2.5% nickel II sulfate, 0.05% 3,3'-diaminobenzidine (DAB) and 0.005% H_2O_2, which yields a dark blue-black reaction product. A separate series of sections was immunostained for rat serum albumin (RSA) without nickel intensification to assess the integrity of the blood-brain barrier (BBB) following the lesion and transplant according to our previously published protocol (Charles et al., 1996). An additional series of sections through the striatum was stained for NADPH-d as described previously (Kordower et al., 1994). Adjacent sections were stained with cresyl violet acetate (pH 3.3) to aid in cytoarchitectonic delineation and to characterize the extent of the lesion. All sections were mounted on gelatin-coated slides, air dried, dehydrated, and coverslipped with Permount.

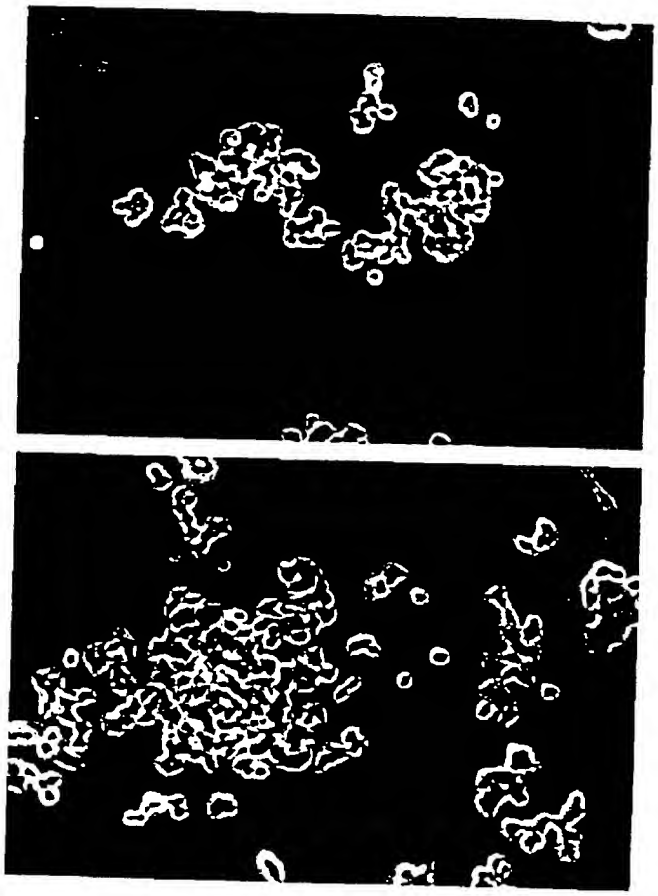

Fig. 1. A: Note the absence of neurite extensions in PC-12 cells exposed for 48h to conditioned media from control stem cells. B: Neurite extensions displayed by PC12a cells following 48h of exposure to conditioned media from control hNGf-secreting stem cells. Scale bar = 50 µm in A,B.

Electron microscopy

Selected DAB-treated sections were rinsed in 0.1 M sodium cacodylate buffer for 15 minutes and placed in 1.0% sodium cacodylate-buffered OsO_4 for 3 hours. After osmication, the tissue was dehydrated in graded ethanols and flat-embedded in plastic (Polybed 812, Ladd). Semi-

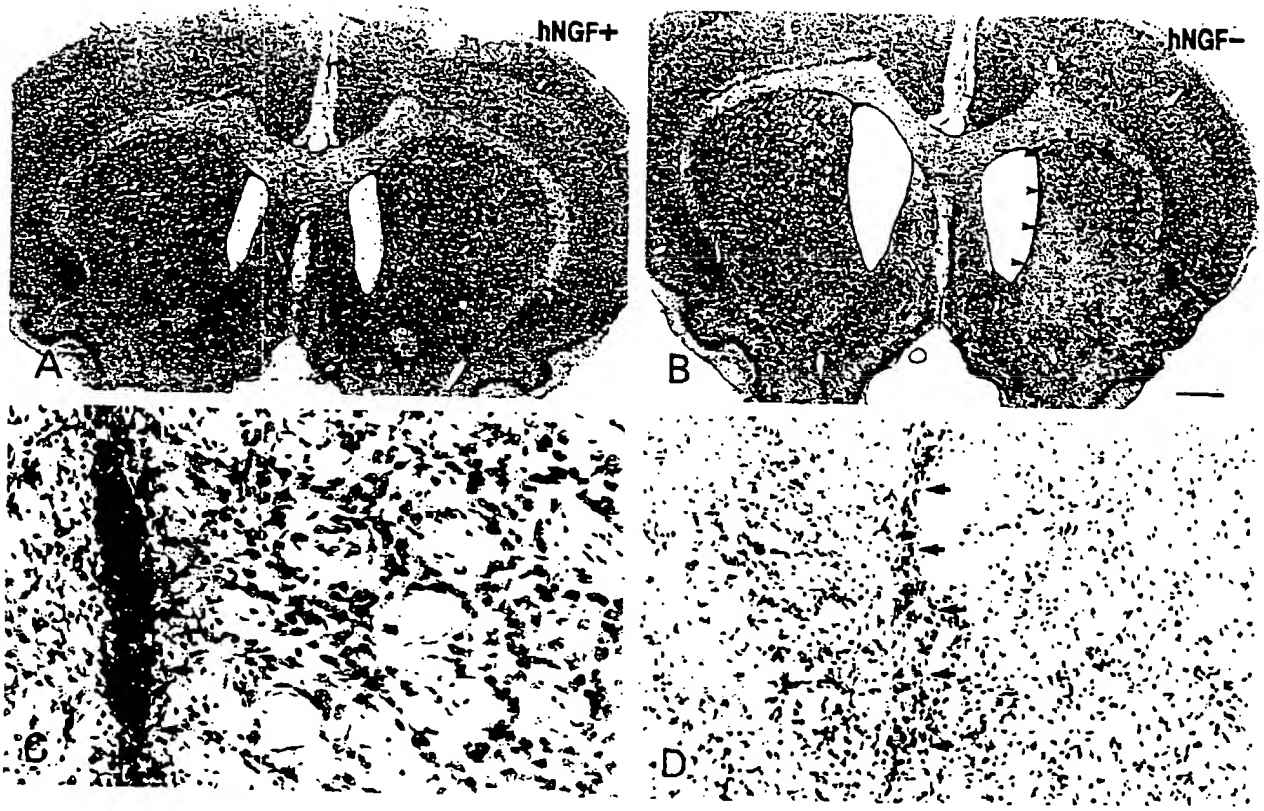

Fig. 2. Low- (A,B) and high- (C,D) power photomicrographs of Nissl-stained sections illustrating the protection of striatal neuronal degeneration by hNGF-secreting stem cell implants. A: Rats receiving hNGF-secreting stem cell implants display minimal or no cell loss following intrastriatal QA injections. B: In contrast, extensive neural degeneration is observed in rats receiving QA lesions and control (non-hNGF-secreting) stem cell grafts. The arrowheads outline the extent of the lesion. C: In hNGF-grafted rats, numerous healthy appearing neurons were observed throughout the striatum even proximal to the QA needle tract (arrows). D: In contrast, virtually no viable neurons and an intense gliosis were observed proximal to the QA needle tract in rats receiving control stem cell implants (arrows). Scale bar = 1,000 μm in A,B; 100 μm in C,D.

thin sections were stained with toluidine blue to verify correct location and orientation. Ultrathin sections (silver-gold interference) were collected on 200-mesh grids and stained with lead citrate and uranyl acetate by conventional methods. The sections were studied with a JOELCO 1200 EX electron microscope.

Immunohistochemistry: Controls and caveats

Controls for immunohistochemistry consist of processing tissue as described above except for using the primary antibody solvent or an irrelevant IgG in lieu of the primary antibody. It is important to note that specificity for any immunocytochemical reaction product is not absolute. Regardless of the fact that substitutions for the primary antibody may abolish immunoreactivity, the potential for antisera to react with structurally related antigens cannot be excluded. Thus, a degree of caution is warranted, and the term immunoreactivity refers to "-like" immunoreactivity.

Quantitative morphometric analysis

The quantitation of striatal neurons was performed as previously described (Emerich et al., 1994, 1996; Kordower et al., 1994) by an investigator blinded to the experimental condition. The size of the lesion was determined by using the NIH Image 1200 analysis system. The rostral caudal extent of the lesion area was traced on equispaced Nissl-stained sections by using a computer mouse, and the lesion area was automatically calculated for that section. Then, the total area was calculated by interpolating the lesion area between measured sections. The present study was concerned with the relative number, not the absolute number, of GAD-ir, ChAT-ir, and NADPH-containing neurons within the intact and lesioned striatum under control and hNGF-grafted conditions. Thus, the number of GAD-ir, ChAT-ir, and NADPH-containing neurons was quantified bilaterally for each animal by using established quantitative morphometric procedures (Emerich et al., 1994, 1996; Kordower et al., 1994). For each rat, five coronal sections through the striatum beginning at the level of the QA injection and extending equidistantly in the anterior and posterior direction at 200-μm intervals were analyzed. Every ChAT-, GAD-, or NADPH-d-positive neuron in each of those sections was counted by an individual blinded to the animal's experimental condition.

Data analyses

Data were analyzed with an SAS-PC™ statistics program. Analyses of variance were conducted by using the procedures for general linear models (SAS Institute Inc.,

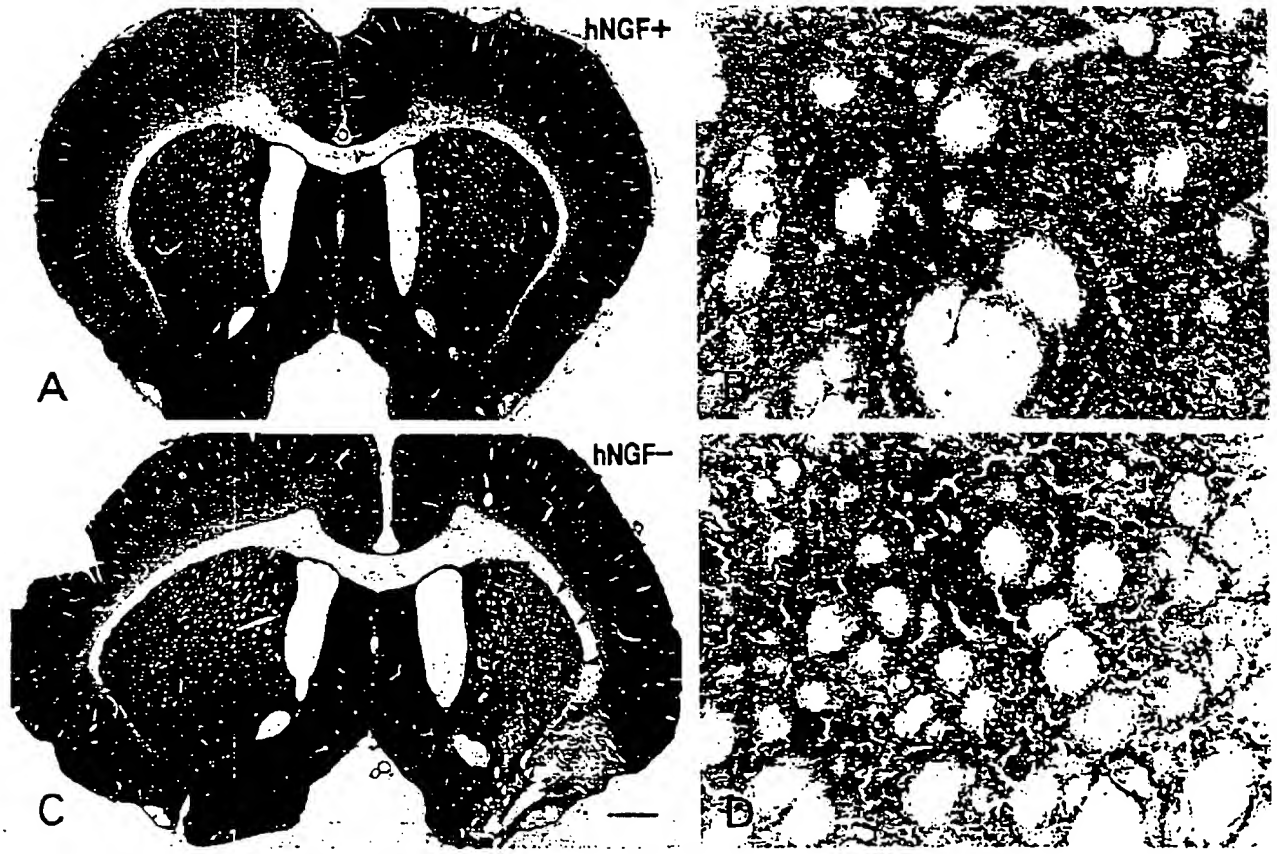

Fig. 3. Low- (A,C) and high- (B,D) power photomicrographs of glutamic acid decarboxylase(GAD)-immunostained sections illustrating the protection of gamma-aminobutyric (GABA)-ergic neurons by hNGF-secreting stem cell implants. A: Note the similarity in GAD-immunoreactive staining pattern on the lesion/graft side of the striatum (right side) relative to the intact contralateral side in hNGF-grafted animals. B: At higher magnification, numerous GAD-ir neurons were observed within the lesioned/hNGF-grafted striatum. C: In contrast, an extensive loss of GAD-ir neurons and neuropil was observed in QA-lesioned rats receiving control stem cell implants. The scope of the lesion is demarcated by arrowheads. D: At higher magnification, few GAD-ir neurons were observed. Scale bar = 1,000 μm in A,C; 50 μm in B,D.

1989). Data are presented in the text and in all figures as means ± SEM.

RESULTS

PC-12 cell bioassay

Conditioned media obtained from the differentiated GFAP-hNGF and control stem cell cultures were collected and cultured with PC-12 cells. Within 24 hours following exposure to the conditioned media, long neurites were observed extending from the PC-12 cells that were exposed to media from hNGF-secreting stem cells (Fig. 1B). In contrast, neurite extension was not induced in PC-12 cells exposed to media from control (non-hNGF secreting) stem cells for up to 48 hours (Fig. 1A).

GFAP-hNGF stem cell grafts: Effects on lesion area

Nissl-stained sections revealed large areas of neural degeneration within the striatum in all animals receiving QA lesions and control (non-NGF-secreting) transplants (Fig. 2B). Occasionally, the lesion extended into the frontal and temporal neocortices. These lesions were spherical or elliptical in shape and in many sections encompassed the entire striatum, which displayed a marked reduction in neurons and an abundance of glial cells (Fig. 2D). In contrast, small or negligible regions of degeneration were seen within the striatum of rats receiving QA lesions and GFAP-hNGF stem cell implants. In fact, no lesion was discerned in Nissl-stained sections in four of the eight rats receiving NGF-secreting stem cell implants (Fig. 2A). In GFAP-hNGF-grafted animals, healthy appearing neurons could be observed immediately adjacent to the QA injection site (Fig. 2C). Quantitative analyses of the lesion area using a one-way ANOVA between groups supported these qualitative observations. The lesion area in rats receiving control transplants was 12.78 mm^3 ± 2.53. In contrast, the lesion area was significantly diminished (1.91 mm^3 ± 1.02) in rats receiving NGF-secreting stem cell implants [$(F1,13) = 17.45; P = 0.001$].

GFAP-hNGF stem cell grafts: Protection of GAD, ChAT, and NADPH-positive neurons

ChAT-ir, GAD-ir, and NADPH-diaphorase-stained sections within the unlesioned, contralateral striatum, revealed a general pattern of labeled perikarya consistent

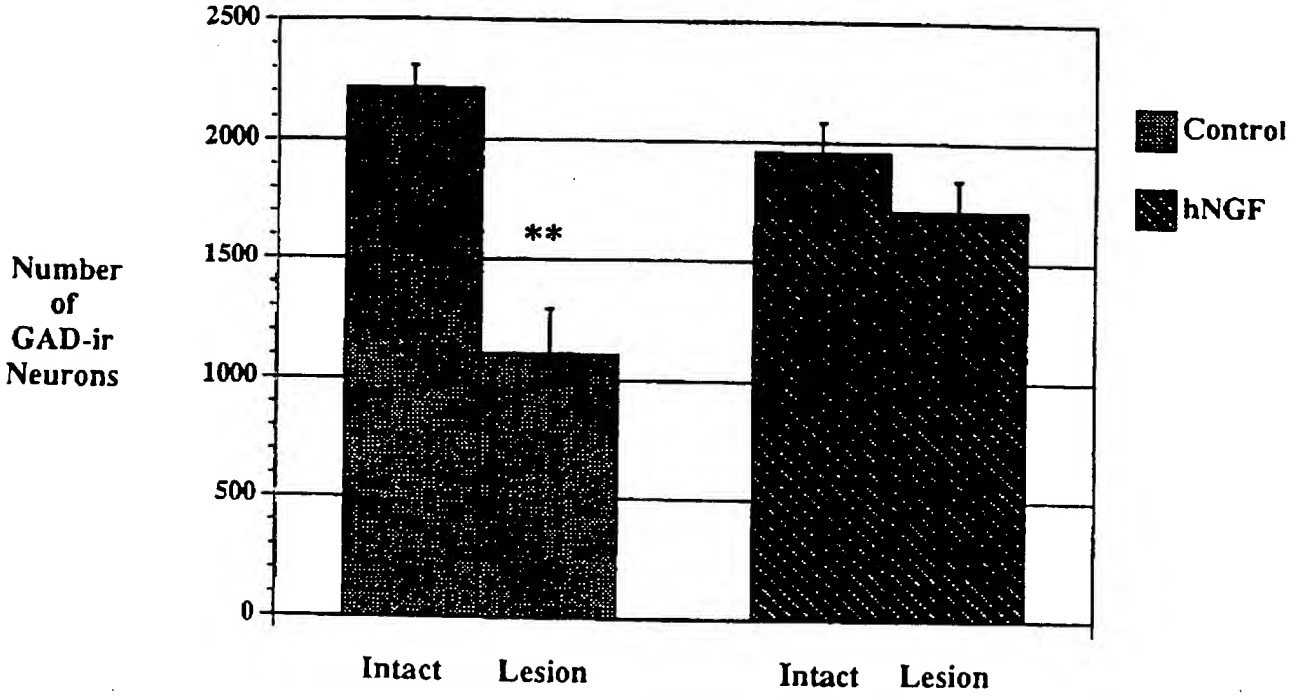

Fig. 4. Quantification of GAD-ir striatal neurons on the intact and lesion side in rats receiving control and hNGF-secreting stem cell implants. $^{**}P < 0.01$.

with previous observations (e.g., Emerich et al., 1994, 1996; Kordower et al., 1994; Martinez-Serrano and Björklund, 1996). All specific staining was eliminated when control experiments were performed with the primary antibody deleted or an irrelevant IgG substituted for the primary antibody.

The ability of hNGF-secreting stem cell implants to prevent the degeneration of GAD-ir, ChAT-ir, and NADPH-d-containing neurons was evaluated with a 2 × 2 ANOVA, including Groups (control vs. NGF stem cells) and Side (Lesioned vs. Nonlesioned) as factors in the analyses. Cell counts for each type of stain (GAD, ChAT, or NADPH-d) were analyzed separately.

In control-grafted rats, there was a dramatic loss of GAD-ir striatal neurons. In sections proximal to the lesion site, there was a substantial loss of GAD-ir neurons (Fig 3C,D). Some sections displayed virtually no GAD-ir striatal neurons ipsilateral to the lesion. In contrast, GAD-ir neurons were spared in GFAP-hNGF stem cell-grafted animals. In a few GFAP-hNGF stem cell-grafted animals, there was no discernible loss of GAD-ir neurons within the striatum at all (Fig. 3A,B). Quantitative analysis confirmed this qualitative assessment. A two-way ANOVA revealed that the number of GAD-positive cells was significantly reduced in all animals on the lesioned side relative to the nonlesioned side; the main effect for Side was statistically significant, $F(1,13) = 48.06$, $P = 0.0001$. However, rats implanted with GFAP-hNGF stem cells displayed an attenuated loss of GAD-ir cell (12.7% vs. 50%) relative to rats implanted with control cells, the Group × Side interaction was statistically significant, $F(1,13) = 19.25$, $P = 0.0007$ (Fig. 4).

The QA lesion resulted in a dramatic loss of ChAT-ir neurons within the striatum in animals receiving control transplants. In sections proximal to the needle tract, there was almost a complete loss of ChAT-ir neurons (Fig. 5B). Those few neurons that did remain following the lesion appeared atrophic with blunted dendrites (Fig. 5D). In contrast, the loss of ChAT-ir cells was completely prevented by intrastriatal transplants of NGF-secreting stem cells. In these animals, there were numerous ChAT-ir neurons throughout the striatum, even in sections that contained the QA lesion needle tract. These neurons were large in size (25–35 μm in diameter) with long neuritic processes, and they displayed the typical morphological profile of healthy cholinergic striatal interneurons (Fig. 5C). Quantitatively, the number of ChAT-positive cells was significantly reduced on the lesioned side relative to the nonlesioned side; the main effect for Side was statistically significant, $F(1,13) = 14.03$, $P = 0.003$. Control-grafted rats displayed a 64.2% reduction in the number of ChAT-ir neurons relative to the contralateral side. This loss was ameliorated in rats implanted with hNGF-secreting cells (−3.2%); the Group × Side interaction was statistically significant: $F(1,13) = 16.76$, $P = 0.001$ (Fig. 6).

In contrast to the potent protection of ChAT-ir and GAD-ir neurons, implants of GFAP-hNGF stem cells had more modest effects upon NADPH-d-positive striatal neurons. On the intact side, numerous small NADPH-d-positive cells with long varicose processes were observed scattered throughout the striatum. In all animals, the number of these cells was substantially reduced on the lesioned side relative to the nonlesioned side; the main effect for Side was statistically significant: $F(1,13) = 92.99$, $P = 0.0001$. Rats receiving control grafts displayed a 84% reduction in NADPH-d positive neurons ipsilateral to the lesion relative to the contralateral side. In many sections, these animals displayed a complete loss of NADPH-d positive cells (Fig. 7C,D). A significant sparing of diaphorase-containing neurons was observed in hNGF- treated

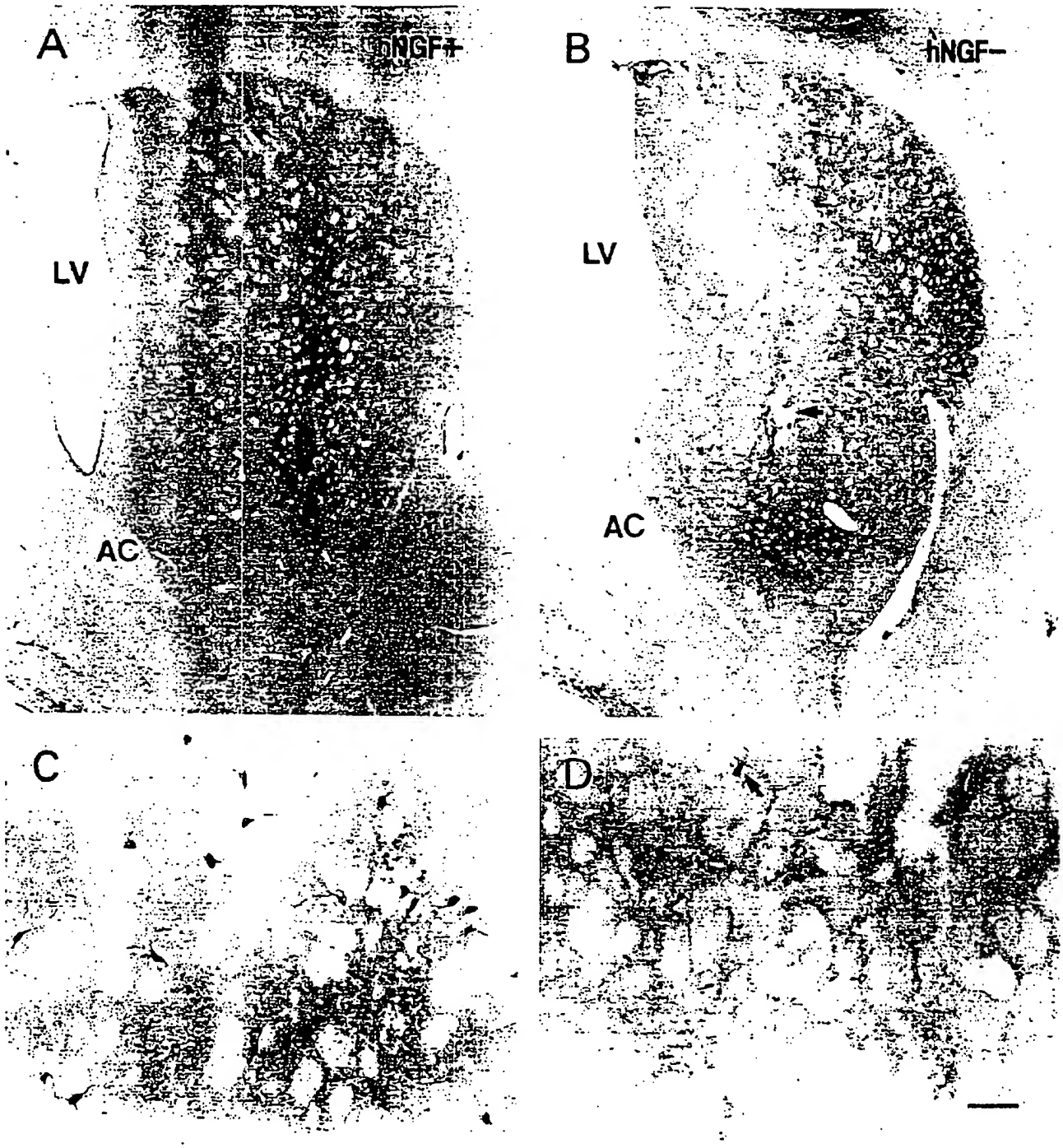

Fig. 5. Low- (A,B) and high- (C,D) power photomicrographs of choline acetyltransferase (ChAT)-immunostained sections illustrating the protection of cholinergic neurons by hNGF-secreting stem cell implants. A: Numerous ChAT-ir neurons scattered throughout the striatum in rats receiving hNGF stem cell implants. B: In contrast, few ChAT-ir neurons were seen in rats receiving control stem cell implants. The arrows in panels A and B illustrate the location of the stem cell implants. This discordance in ChAT-ir staining patterns is not due to technical factors because similar staining patterns are seen in both hNGF- and control-grafted animals within the horizontal limb of the diagonal band. C: In hNGF-grafted rats, ChAT-ir neurons appeared healthy with normal striatal morphological profiles. D: In control-grafted rats, only an occasional shrunken ChAT-ir neuron was observed (arrow). LV, lateral ventricle; AC, anterior commissure. Scale bar = 500 μm in A,B; 100 μm in C,D.

rats; the Group × Side interaction was statistically significant: $F(1,13) = 11.18$, $P = 0.005$; Fig. 8). However, these rats still displayed a 41.4% loss of NADPH-d positive cells. Within the striatum of these animals, a clearly defined locus of the lesion could be discerned (Fig. 7A). In the lesion area, few NADPH-d-positive cells were seen. How-

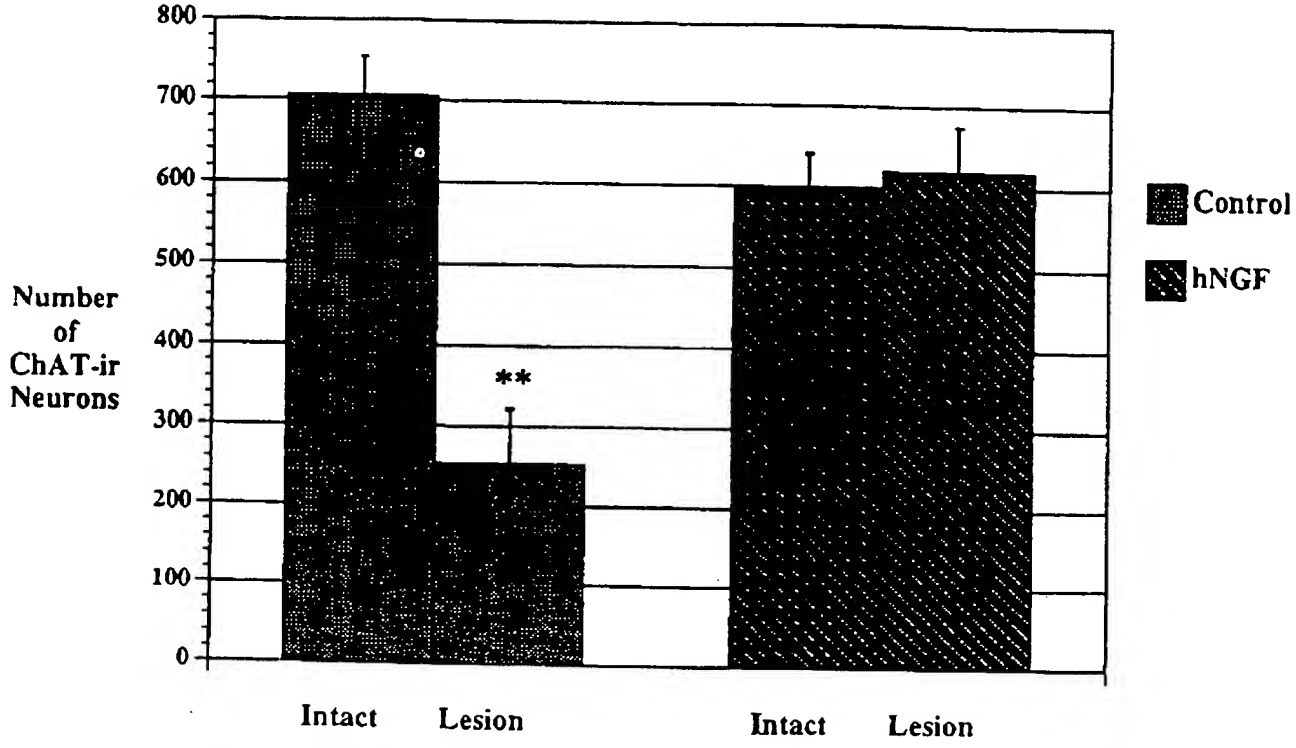

Fig. 6. Quantification of ChAT-ir striatal neurons on the intact and lesion side in rats receiving control and hNGF-secreting stem cell implants. $^{**}P < 0.01$.

ever, once outside the penumbra of the lesion, the NADPH-d stained striatum appeared normal with numerous multipolar neurons giving rise to a dense neuropil (Fig. 7B).

GFAP-hNGF stem cell grafts: Localization of stem cells

A mouse-specific antibody was used as a species-specific marker for the grafted stem cells. By using this marker, grafted stem cells were identified in all control and hNGF-treated animals (Fig. 9A). There were no apparent differences in the extent of graft viability between the two groups. Dense clusters of M2-ir stem cells were observed, principally within and around the needle tract. Little, if any, migration of M2-ir cells was noted. This antibody predominantly stains the external cell membrane, and as such, perikarya are usually not visualized. Rather in most instances, processes were visualized emanating from a nonimmunoreactive perikaryon (Fig. 9B). The exception to this staining pattern was observed principally at the base of injection sites where a dense column of cells within the tract expanded into a teardrop-shaped or round cluster of cells. Under these conditions, M2 immunoreactivity appeared to be present within grafted perikarya (Fig. 9C). With Nissl stains, these graft deposits appeared as collections of small pale perikarya with a darkly stained nucleolus (Fig. 9D). Surrounding these cells were smaller more darkly stained cells that exhibited morphological profiles similar to glial cells. M2-immunoreactive sections counterstained for Nissl substance indicate that many, but not all, of these more darkly stained cells were of graft origin.

GFAP-hNGF stem cell grafts: Sprouting of cholinergic basal forebrain fibers

Stem cells derived from transgenic mice in which the GFAP promoter directs the expression of hNGF induced a $p75^{NTR}$-ir sprouting response (Fig. 10). This phenomenon was seen in all animals receiving hNGF-secreting stem cells (Fig. 10A–C) but was never observed in control rats treated identically except that the grafted stem cells lacked the hNGF construct (Fig. 10D). In animals grafted with non-hNGF-secreting stem cells, only a rare $p75^{NTR}$-ir process was observed (Fig. 10D). In rats receiving hNGF-secreting stem cell grafts, fibers immunoreactive for the low-affinity $p75^{NTR}$ were seen surrounding the grafted cells and penetrating for short distances within the graft site. These fibers were thin and varicose with a CNS morphology. These fibers did not stain for DβH (data not shown), ruling out the possibility that this innervation could result from ingrowth of sympathetic fibers that are also immunoreactive for the $p75^{NTR}$. Rather, these fibers appeared to emanate from cholinergic neurons within the basal forebrain, a region whose neurons are exquisitely sensitive to the trophic and tropic influences of NGF. The $p75^{NTR}$-immunostained fibers were seen coursing from the underlying horizontal limb of the diagonal band of Broca and the anterior subdivision of the nucleus basalis toward the hNGF-secreting graft sites following an aberrant trajectory that coursed through the ventral striatum. These fibers surrounded the graft along the periphery of its entire dorsoventral extent and penetrated the graft for short distances. Although tract tracing would provide direct evidence supporting the observation that this $p75^{NTR}$

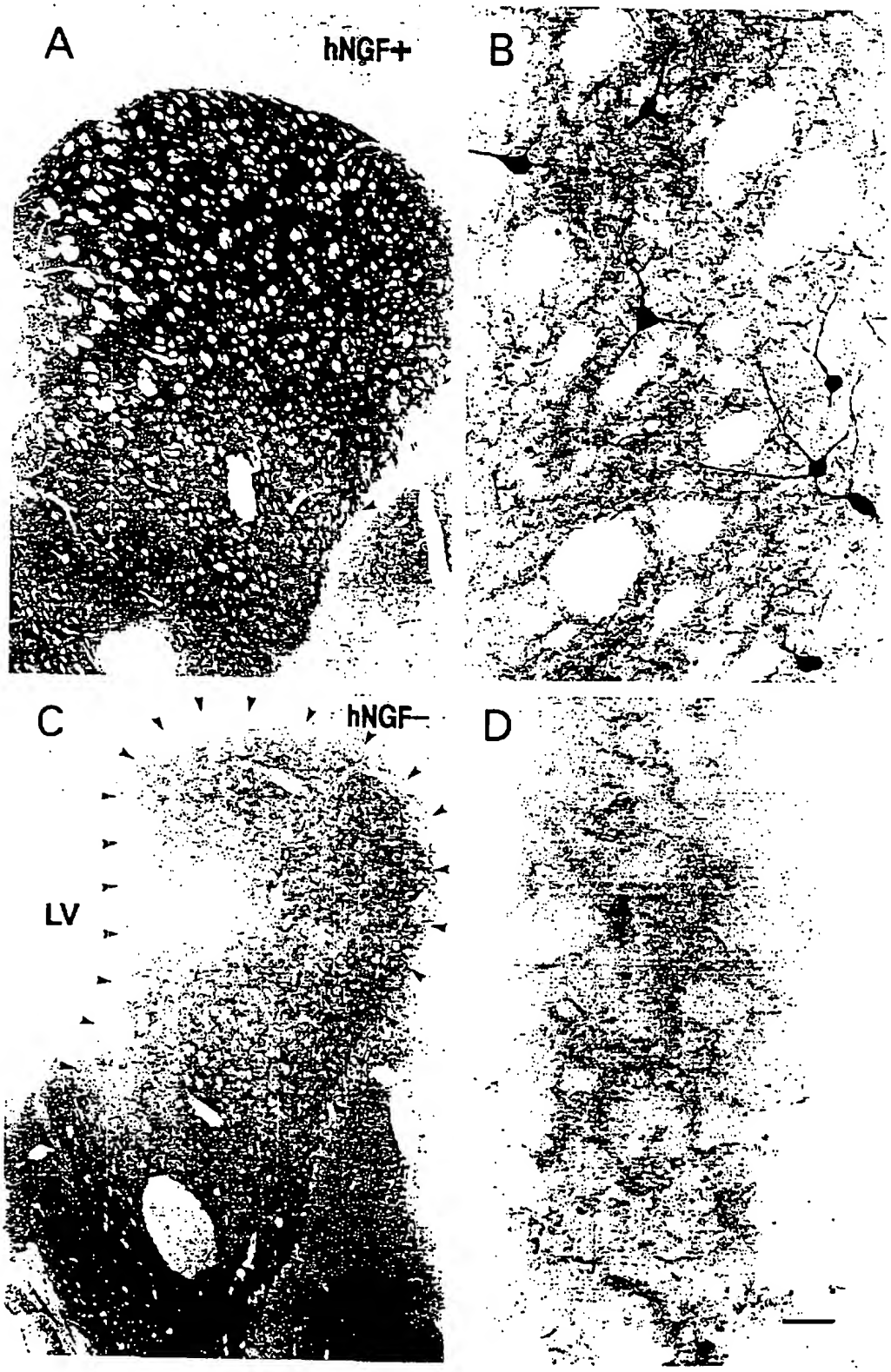

Fig. 7. Low- (A,C) and high- (B,D) power photomicrographs of NADPH-d-stained sections illustrating the protection of diaphorase-containing neurons by hNGF-secreting stem cell implants. **A:** In hNGF-grafted rats, there was a partial loss of NADPH-d-containing cells and neuropil (arrowheads). **B:** The NADPH-positive cells outside this area appeared healthy with normal morphological profiles. **C:** In contrast, QA-lesioned rats receiving control stem cell implants displayed a comprehensive loss of NADPH-d-positive neurons, which often encompassed the entire striatum (arrowheads). **D:** At higher magnification, few viable NADPH-d-stained neurons were observed. Scale bar = 500 μm in A,C; 50 μm in B,D.

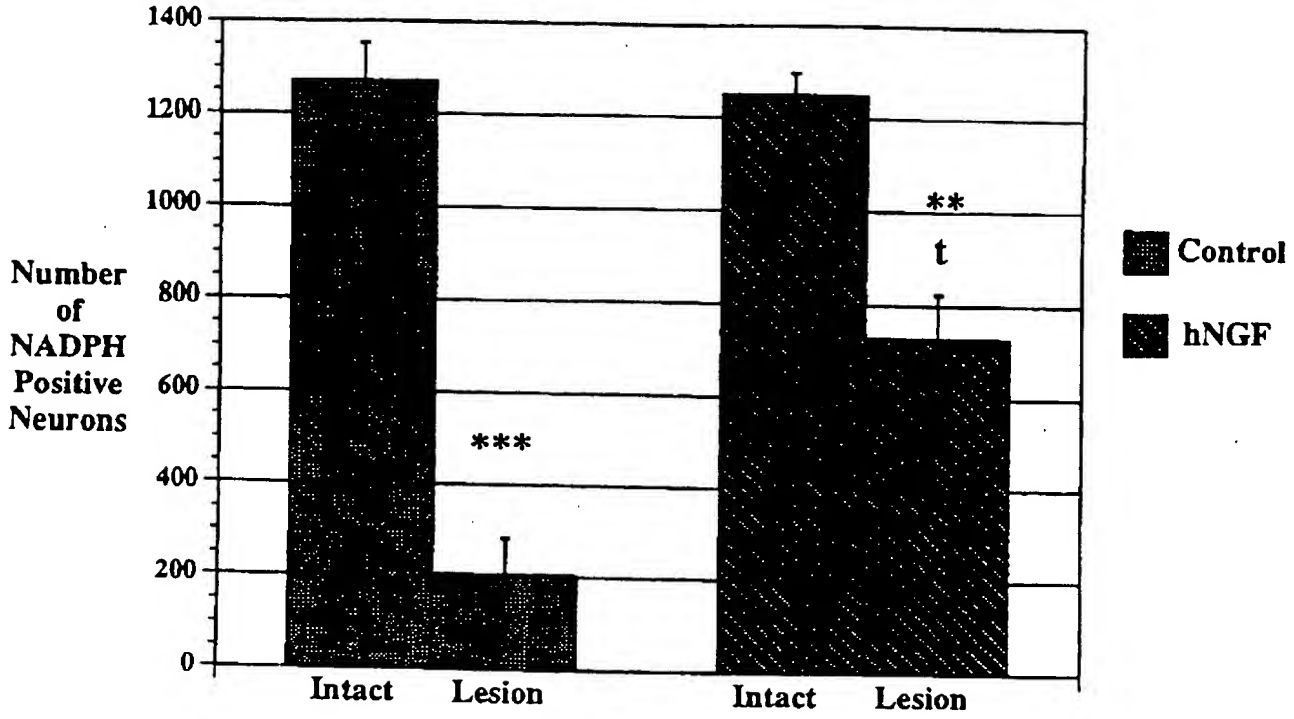

Fig. 8. Quantification of NADPH-positive striatal neurons on the intact and lesion side in rats receiving control and hNGF-secreting stem cell implants. ***$P < 0.001$ relative to the intact side; **$P < 0.01$ relative to the intact side; t, $P < 0.01$ relative to the lesion side of control-grafted rats.

sprouting response emanates from the basal forebrain, it is notable that $p75^{NTR}$ is a specific marker for cholinergic basal forebrain neurons in the rat forebrain (e.g., Kordower et al., 1988). $p75^{NTR}$ is not found within the striatum under normal conditions and is not induced in neurons within the striatum following mechanical damage, excitotoxic lesions, or intrastriatal administration of NGF (Bartus et al., 1996).

GFAP-hNGF stem cell grafts: inhibition of glial and barrier changes

In both nongrafted rats and rats receiving implants of control stem cells, injections of QA into the striatum resulted in a vigorous, but nonuniform expression of $p75^{NTR}$ receptor. Upon cursory light microscopic examination, this staining pattern appeared to be within vascular elements (Fig. 11). However, electron microscopy revealed that the immunoexpression was exclusively associated with astrocytes (see below). This response was massive, often involving the entire striatum and surrounding cerebral cortex. In some animals, the $p75^{NTR}$ response spread into the lateral septum. This response was only observed ipsilateral to the QA injection and was never observed on the intact side. The magnitude of this response was similar in ungrafted rats and rats receiving control stem cell grafts, indicating that the presence of the grafted cells did not underlie this response. Astroglial elements upon vessels throughout the lesioned striatum were densely immunoreactive for the $p75^{NTR}$ receptor (Fig. 11B–E). In addition to the vasculature-related astroglia, occasional triangular shaped cells of unknown origin, possibly microglia, were also immunoreactive for the $p75^{NTR}$ receptor within the sphere of the lesion (Fig. 11E). Remarkably, this robust $p75^{NTR}$ vascular and cellular response following QA was virtually absent within the striatum of rats receiving identical lesions and stem cell grafts with the addition of the hNGF construct. In all but one of these animals, the striatum appeared normal, although some animals displayed an attenuated cellular response within the overlying cerebral cortex. It is notable that the one animal that did display a modest $p75^{NTR}$-immunoreactive vascular response within the striatum displayed the least neuronal protection from the hNGF stem cell grafts supporting the concept that this response is associated with the neuronal death resulting from the QA lesion. Extravasated RSA, which was found specifically within the QA lesioned striatum (Fig. 12B), was not observed within the striatum in hNGF-grafted rats, indicating an intact blood-brain barrier was sustained within the striatum of these animals (Fig. 12A). Occasionally, there was RSA-ir within the overlying cortex in hNGF-grafted rats.

As was suggested by the light microscopic immunocytochemical data, ultrastructural analyses revealed that the immunoexpression of $p75^{NTR}$ varied in relation to the vascular network within the lesioned area. The deposition of reaction product was always associated with astrocytic membranes and surrounding extracellular space and not the cerebral endothelia or their basement membranes. There was no evidence that the $p75^{NTR}$ immunoreactivity was directly related to the cerebral endothelia or perivascular space. Sometimes, the reaction product would appear at the interface between an astrocyte cell body and a foot process adjacent to a capillary (Fig. 13A,B). Most often, however, extensive $p75^{NTR}$ immunoexpression was associated with the membranes of reactive, filament-laden, astroglial processes. At times, the reactionproduct ex-

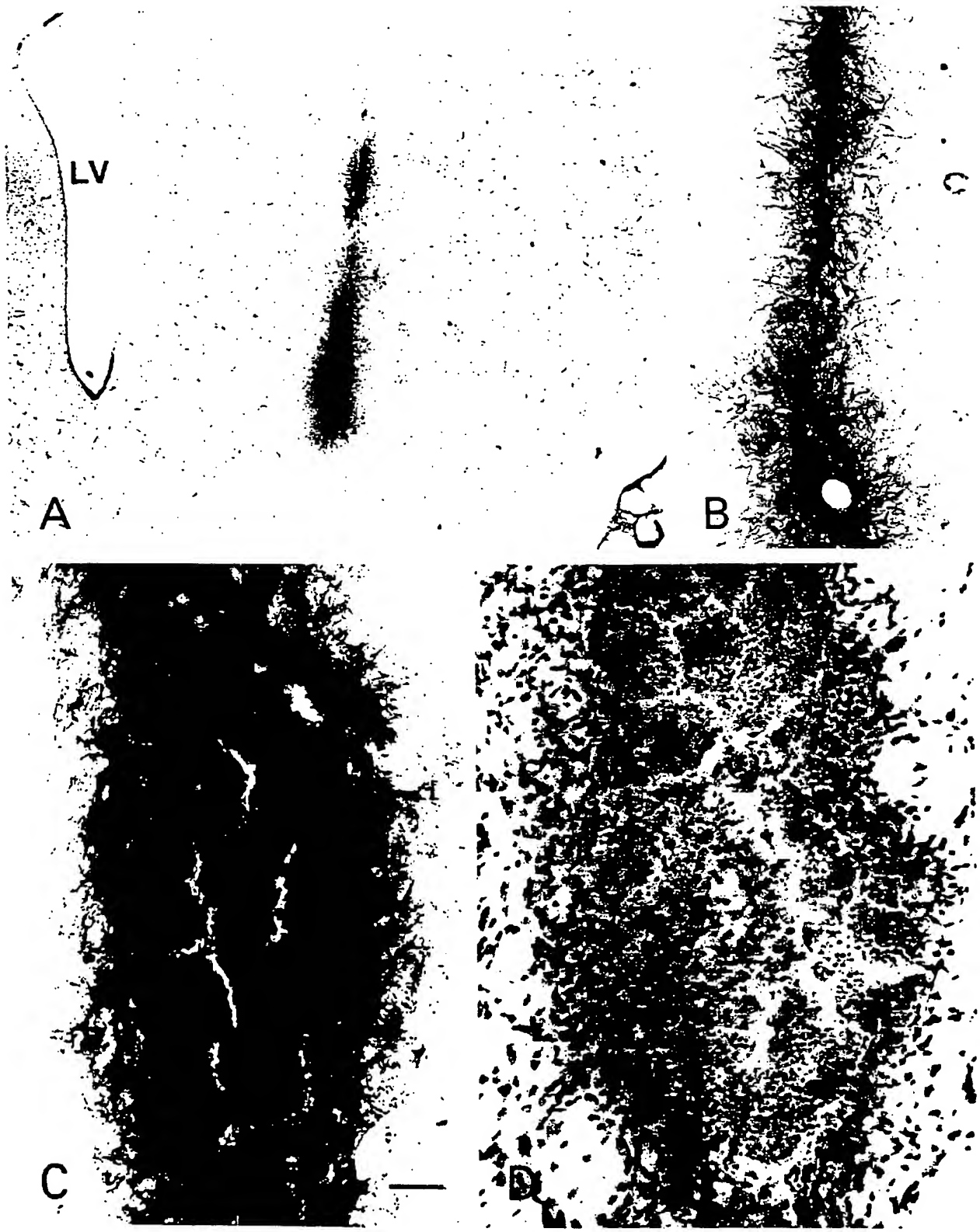

Fig. 9. Low- (A) and medium- (B) power photomicrographs of stem cells visualized with the mouse-specific neurofilament antibody M2 in an hNGF-grafted animal. Note the exclusive presence of stained cells within the site of graft deposits. C: High-power photomicrograph of M2-ir graft site illustrating a dense collection of grafted cells. D: A Nissl-stained section adjacent to the one illustrated in panel C illustrates the center of the graft containing palely stained cells that display an immature stem cell-like morphology. In contrast, cells at the borders of the graft exhibit a glial morphology. LV, lateral ventricle. Scale bar = 500 μm in A, 250 μm in B, 50 μm in C,D.

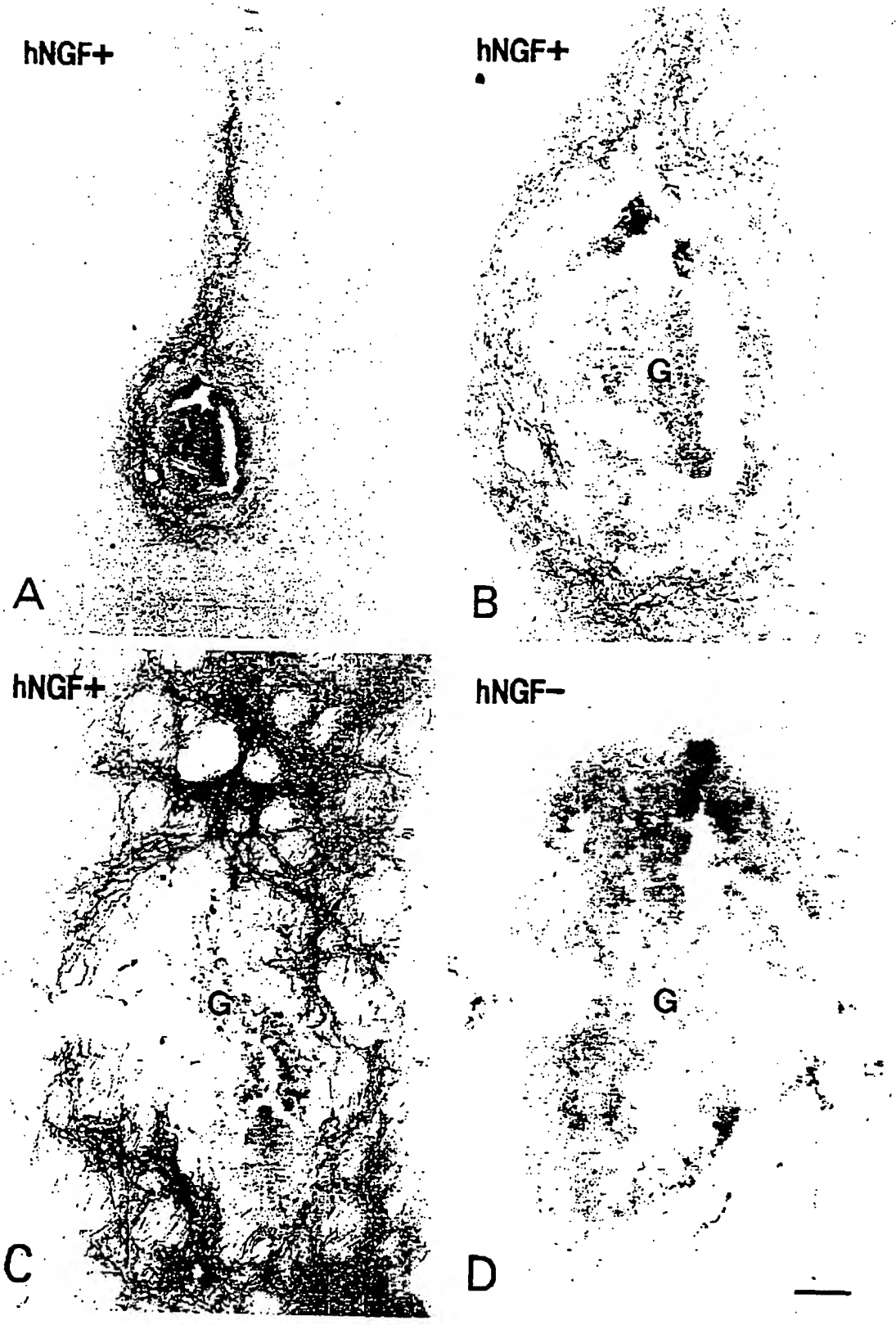

Fig. 10. Low- (A) and high- (B–D) power photomicrographs of sections immunostained for $p75^{NTR}$. A–C illustrated that a plexus of $p75^{NTR}$-ir fibers encircles and penetrates into hNGF-secreting stem cell grafts (G). (D) In contrast, only an occasional $p75^{NTR}$-ir process is seen coursing near a control, non-hNGF-secreting, stem cell graft (G). Scale bar = 250 μm in A, 100 μm in B–D.

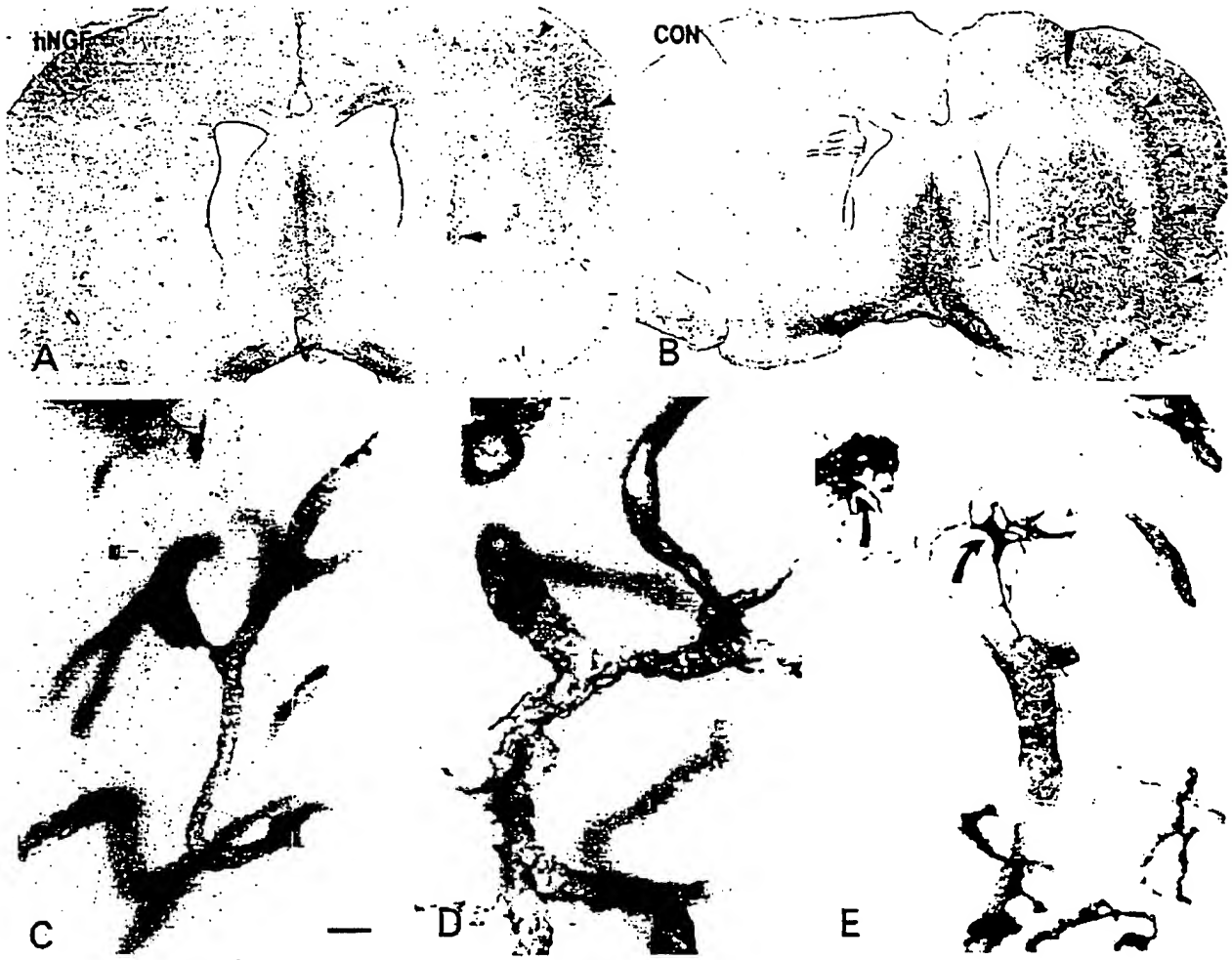

Fig. 11. The hNGF-secreting stem cell implants prevented a QA-lesioned mediated $p75^{NTR}$-ir response within the striatum. A: Note the absence of $p75^{NTR}$-ir staining within the striatum of hNGF-grafted rats except for a small area around the implant site (arrow) that is composed of sprouting fibers from the basal forebrain. $p75^{NTR}$-ir was occasionally seen within the cortex of these animals only on the side ipsilateral to the lesion (arrowheads). B: In contrast, a massive $p75^{NTR}$-ir glial response around vessels was seen within the striatum and cortex (arrowheads) in QA-lesioned rats receiving no implant or (C–E) control stem cell grafts. Note C, a nonuniform expression $p75^{NTR}$-ir within astroglia surrounding the blood vessels. E: High-power photomicrographs illustrating the expression of glial-derived $p75^{NTR}$-ir upon striatal microcapillaries (curved arrows). Scale bar = 1,000 μm in A,B, 10.3 μm in C,D, 15.6 μm in E.

tended between a large process and a thin foot process adjacent to capillaries or larger microvessels (Fig. 13C,D). Although the vascular basement membranes were always free of $p75^{NTR}$ immunoreactivity, by contrast the reaction product for RSA completely flooded the basement membranes and perivascular spaces (data not shown).

DISCUSSION

The present study clearly shows that implants of EGF-responsive stem cells, which upon differentiation secrete hNGF, forestall the degeneration of striatal neurons destined to die in an animal model of HD. The use of stem cells that secrete a desired bioactive molecule to modify degenerating host systems has specific advantages over other forms of cellular delivery. These cells can be exponentially expanded in vitro without the use of oncogenes providing a limitless supply of donor cells. They can be banked and screened for the absence of adventitious agents. These cells also have the properties of self-renewal and under appropriate conditions differentiate into specific phenotypes based upon their local environment.

GFAP-hNGF stem cell grafts: Effects on striatal neuronal populations

In the present study, grafts of EGF-responsive stem cells that secrete hNGF clearly reduce the size of a striatal lesion resulting from injections of a high dose (225 nmol) of QA. In some animals, there was no discernible loss of neurons on Nissl-stained sections. Critically, the present study shows that the loss of GABA-ergic neurons, which provide the major outflow of information emanating from the striatum, was completely prevented in rats receiving hNGF-producing grafts. Previous studies using NGF-secreting fibroblasts have shown that the size of the lesion is reduced following grafts of hNGF-producing fibroblasts (Schumacher et al., 1991; Emerich, 1994; Frim et al.,

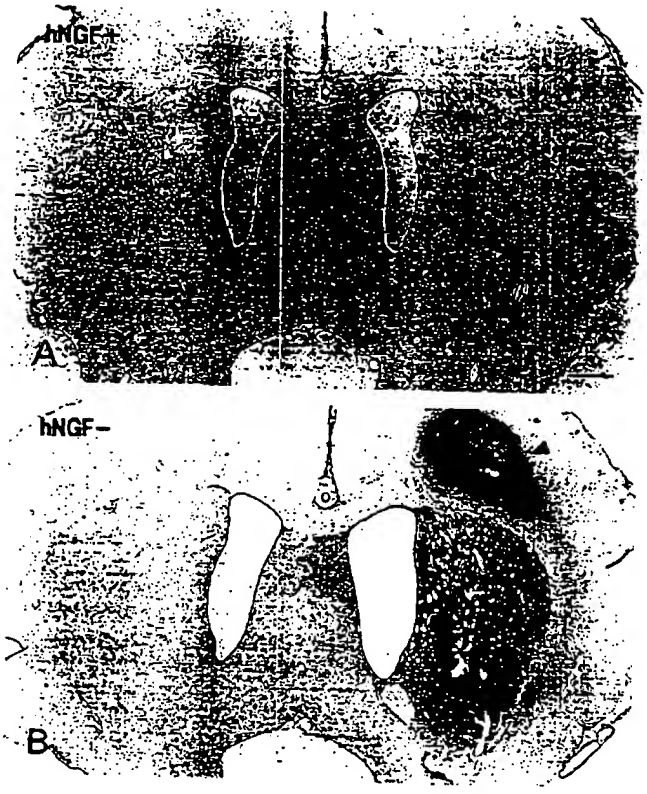

Fig. 12. Low-power photomicrographs of rat serum albumin (RSA)-immunostained sections. A: In hNGF-grafted animals, there was virtually no expression of RSA even at the site of the QA injection (arrow) illustrating an intact blood-brain barrier. B: In contrast, rats receiving control stem cell implants displayed a massive expression of RSA-ir throughout the striatum and within the overlying cortex (arrowhead). Scale bar = 1,000 μm in A, B.

1993a,b,c, 1994). This would suggest that GABA-ergic cells, which comprise between 95 and 99% of striatal interneurons, are protected. Furthermore, Martinez-Serrano and Björklund (1996) used DARPP-32 immunohistochemistry to illustrate that grafts of NGF-producing progenitor cells sustain the GABA-ergic innervation to the globus pallidus in QA-lesioned rats in addition to protecting striatal projection perikarya. Taken together, these three lines of evidence serve to generate a consensus that grafts of hNGF-producing cells can prevent the degeneration of GABA-ergic neurons and sustain striatal circuitry in a rodent model of HD. Additionally, the NGF stem cell grafts prevented the loss of a number of other striatal neuronal population including cholinergic cells and cells containing the enzyme NADPH-d. These cell populations are not typically vulnerable in the excitotoxic lesion model or in HD. However, we used a very high dose of QA (225 nmol) in this study, which resulted in the degeneration of these additional cell populations.

Neuroprotection of striatal neurons by trophic factors: Issues of trophic factor delivery

Interestingly, there appears to be a dichotomy about the neuronal populations protected by NGF depending on the method of neurotrophin delivery. Infusions of recombinant NGF spares only cholinergic striatal interneurons destined to degenerate following excitotoxic lesions of the striatum (Davies and Beardsall, 1992; Kordower et al., 1994; Venero et al., 1994). Similarly, intrastriatal infusion of NGF specifically induces hypertrophy of cholinergic neurons (Gage et al., 1989; Bartus et al., 1996) and specifically increases ChAT mRNA (Venero et al., 1994) in intact animals. These findings are not surprising because immunohistochemical studies have shown that the trkA receptor, which transduces the NGF signal, is located exclusively within cholinergic interneurons in the striatum (Steinenger et al., 1993; Sobreviela at al., 1994; Chen et al., 1996). Because cholinergic degeneration is not a central pathology in HD, sparing these neurons would likely not be a useful treatment strategy. However, NGF delivered under differing conditions can effect a number of seemingly trkA-negative neuronal populations. Mattson and co-workers show that in vitro application of NGF prevents the excitotoxic degeneration of non-trkA-expressing hippocampal neurons resulting from glucose deprivation or hypoxia (Cheng and Mattson, 1992; Cheng et al., 1992, 1993; Mattson et al., 1993; Mattson and Cheng, 1993; Mattson and Scheff, 1994). Recently, a number of studies have shown that transplants of NGF-secreting fibroblasts rescue cholinergic and noncholinergic striatal neurons from excitotoxic degeneration (Schumacher et al., 1991; Emerich, 1994; Frim et al., 1993a,b,c, 1994). Moreover, hNGF-secreting fibroblasts induce hypertrophy in both cholinergic and noncholinergic striatal neurons in intact animals (Kordower et al., 1996). These findings gave rise to the hypothesis that cellular delivery of NGF plus an additional factor secreted by fibroblasts combined to provide a novel sphere of trophism that included both cholinergic and noncholinergic striatal neurons (Kordower et al., 1996). However, the present study and the recent findings of Martinez-Serrano and Björklund (1996) indicate that grafts of stem cells that have been genetically modified to secrete NGF also prevent the degeneration of GABA-ergic and NADPH-expressing striatal neurons, in addition to cholinergic cells. It remains likely that the protection of ChAT-positive neurons in this and previous studies using cellular delivery of NGF results from the neurotrophin binding to trkA receptors located upon cholinergic neurons. However, the mechanism by which NGF-secreting grafts provide trophic support for noncholinergic, non-trkA-expressing cells remains elusive.

Neuroprotection of striatal neurons by trophic factors: Potential mechanisms of action

One potential explanation is that NGF-secreting cells increase the production of free radical scavengers, such as catalase (Frim et al., 1994), which then protects striatal neurons in a general and nonspecific fashion. If this hypothesis is correct, then infusions of NGF, which only provide trophic support for cholinergic interneurons, should not induce catalase expression. This important piece of information remains to be determined. An alternative hypothesis stems from the observation in the present study that hNGF-secreting grafts prevent the expression of $p75^{NTR}$ receptor-ir within or upon the membranes of apparently reactive astrocytes following excitotoxic lesions of the striatum. Although this appears to be the first report of vascular $p75^{NTR}$ immunoreactivity following excitotoxic lesions of the striatum, similar changes in other regions

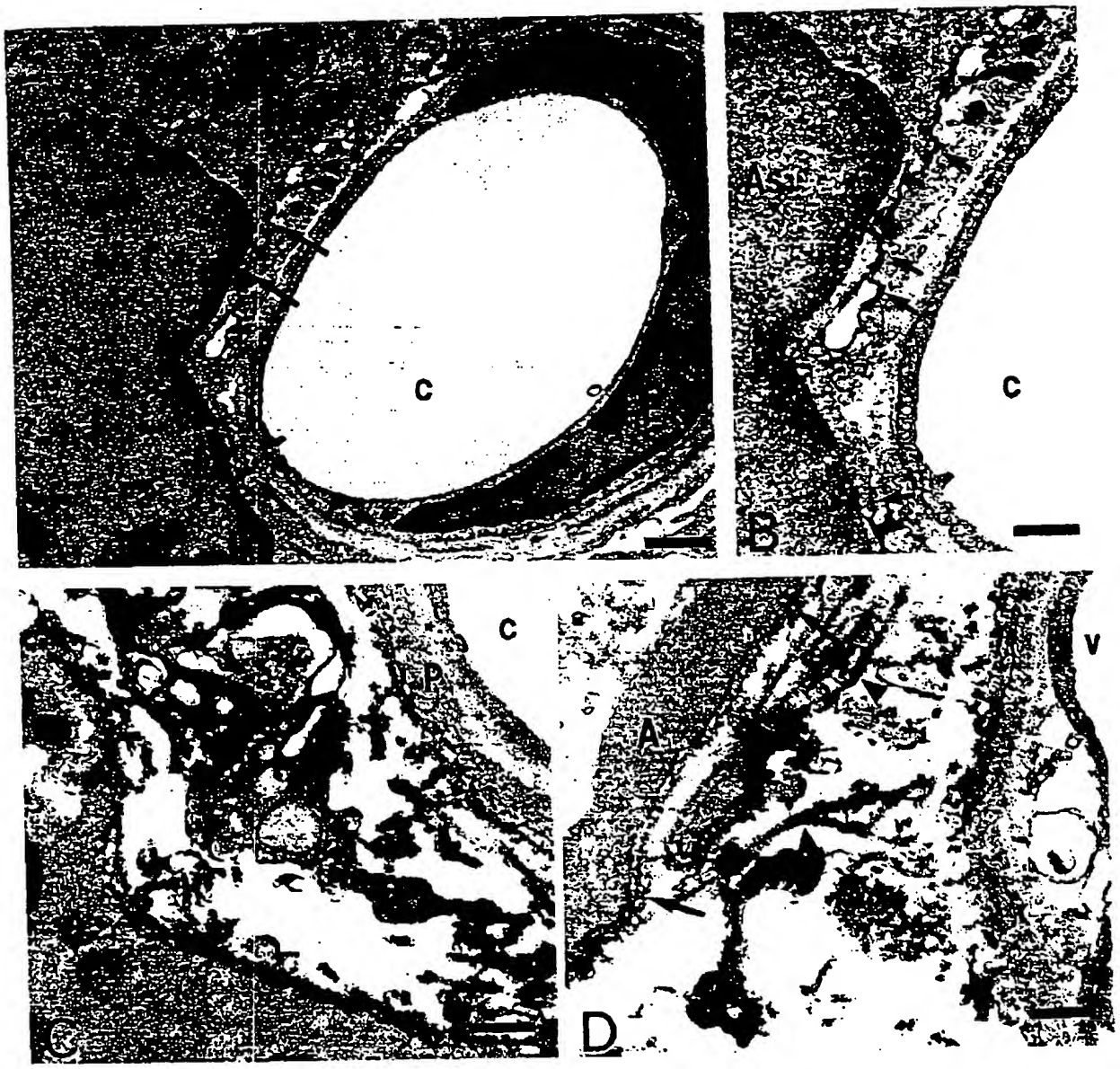

Fig. 13. A: Astrocyte cell body (As) is in close proximity to a cerebral capillary (c) in which the chromatin distribution of the endothelial cell (E) is suggestive of cell division. Arrows denote $p75^{NTR}$ immunoexpression on astrocyte cell membrane. B: Higher magnification of Figure 1 showing discrete deposition (arrows) of $p75^{NTR}$ immunoexpression between the astrocyte soma (As) and an astrocyte foot process (P) adjacent to the capillary (c). C: Extensive $p75^{NTR}$ immunoexpression is present in the interval between a large reactive astrocytic process (A) and a thin foot process (P) adjacent to a capillary (c). Note that the reaction product is found outside the basement membrane (*) surrounding the capillary. D: Strong $p75^{NTR}$ immunoexpression is present along a reactive astroglial (A) process (arrows) and also coats some collagen fibrils (arrowheads) adjacent to a larger microvessel (v). Again, the reaction product is not associated with the endothelium or its basement membrane (*). Scale bars = 0.85 μm in A, 0.4 μm in B, 0.35 μm in C,D.

have been reported in the dorsal column nuclei following excitotoxic lesions of the ventrobasal complex of the thalamus (Junier et al., 1994). In ungrafted rats or rats receiving stem cells that were not genetically modified to secrete hNGF, an extensive network of $p75^{NTR}$-ir astroglial processes related to the vascular network was observed specifically within the grafted striatum. The blood-brain barrier was clearly compromised as shown by the extensive leakage of rat serum albumin within the striatal parenchyma ipsilateral to the lesion. This effect was prevented in hNGF-grafted animals. Interestingly, some rats treated with hNGF-secreting stem cells still showed this $p75^{NTR}$-ir astroglial response in the overlying cortex, a region that would not be expected to receive graft-derived hNGF from cells grafted to the striatum. It appears that this response is related to neural degeneration because it was observed in the one hNGF-grafted rat that displayed the least neural protection. Recently, it has been postulated that naked $p75^{NTR}$ unbound to NGF kills cells (Rabizadeh et al., 1993). Whether the cell death observed

in ungrafted or control-grafted rats following QA and its prevention by graft-derived hNGF is due in part to this striatal expression of $p75^{NTR}$ upon glial elements deserves further scrutiny. Because QA lesions resulted in an opening of the BBB, it remains possible that some aspects of neural degeneration associated with QA toxicity may result from secondary factors originating from the systemic circulation in addition to its well-established excitotoxic mechanisms. Moreover, the barrier dysfunction was directly associated with an upregulation of $p75^{NTR}$ on reactive astroglia. By preventing the astroglial/$p75^{NTR}$ response, the hNGF-secreting grafts appear to prevent the breakdown of the blood-brain barrier, possibly through an astroglial-mediated mechanism. This mechanism may underlie the protection of noncholinergic, trkA-negative neurons, by hNGF-secreting stem cell grafts. These findings could be a fertile area for future investigations of neural protection and blood-brain barrier function.

Future directions

For a gene therapy approach to be of value for the treatment of a neurodegenerative disease, such as HD, the long-term expression of the appropriate transgene is critical. In the present study, we observed the potent viability of grafted stem EGF-responsive cells for up to 3 weeks by using a species-specific marker. Recently, we used immunohistochemical techniques to determine that these cells continue to express hNGF for up to 3 weeks at a level sufficient to sustain neuronal hypertrophy in intact animals (Carpenter and Kordower, unpublished data). Additionally, the hNGF-mediated sprouting response was robust 3 weeks posttransplantation, providing circumstantial evidence that hNGF was still being secreted by grafted cells. However, long-term studies are needed to show conclusively that hNGF continues to be secreted by these cells in vivo.

The present study clearly shows that intrastriatal implants of hNGF-secreting stem cells protect vulnerable populations of striatal neurons from excitotoxic degeneration in a rodent model of HD. The magnitude of this effect is similar to what we have recently seen following implants of hCNTF-producing fibroblasts (Emerich et al., 1996). The use of trophic factors in a neural protection strategy may be particularly relevant for the treatment of HD. Unlike other neurodegenerative diseases, genetic screening can identify virtually all individuals at risk that will ultimately suffer from HD. This provides a unique opportunity to design treatment strategies that can intervene prior to the onset of striatal degeneration. Thus, instead of replacing neuronal systems that have already undergone extensive neuronal death, trophic factor strategies can be designed to support host systems programmed to die later in the organism's life. Studies assessing the long-term benefits of this approach in rodents and nonhuman primate models of HD, in conjunction with behavioral studies aimed at evaluating the effects of trophic factor-secreting transplants upon both motor and cognitive sequelae resulting from excitotoxic striatal lesions, will ultimately determine the clinical utility of this novel approach. Additionally, when evaluating a novel therapeutic strategy, the impact of unwanted effects of the intervention need to be considered. In the present study, sprouting of cholinergic fibers from the nucleus basalis to the NGF stem cells was observed. Whether this aberrant sprouting would occur in the large primate brain where the striatal target and the NGF-responsive basal forebrain cells are spatially more segregated and whether this sprouting response is functionally deleterious needs to be established.

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Human neural stem cells improve cognitive function of aged brain

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The capability for *in vitro* expansion of human neural stem cells (HNSCs) provides a well characterized and unlimited source alternative to using primary fetal tissue for neuronal replacement therapies. The HNSCs, injected into the lateral ventricle of 24-month-old rats after *in vitro* expansion, displayed extensive and positional incorporation into the aged host brain with improvement of cognitive score assessed by the Morris water maze after 4 weeks of the transplantation. Our results demonstrate that the aged brain is capable of providing the necessary environment for HNSCs to retain their pluripotent status and suggest the potential for neuroreplacement therapies in age-associated neurodegenerative disease. *NeuroReport* 12:1127–1132 © 2001 Lippincott Williams & Wilkins.

Key words: Aging; Differentiation; Engraftment; Memory; Migration; Progenitor; Transplantation

INTRODUCTION

The discovery of multipotent neural stem cells (NSCs) in the adult brain [1,2] has wrought revolutionary changes in the theory on neurogenesis, a theory that now suggests that regeneration of neurons can occur throughout life. To further this revolution, we have recently shown that human neural stem cells (HNSCs) differentiated and survived >3 weeks in basal media without the addition of any supplements or exogenous factors [3]. This result suggests that HNSCs are capable of producing endogenous factors necessary for their own survival and neuronal differentiation. Together, these recent findings stimulated us to investigate the transplantation of HNSCs to determine whether or not the aged brain will provide the necessary environment needed for a successful HNSC transplantation. Here we show, for the first time to our knowledge, that not only did HNSCs expanded *in vitro* survive 30 days after xenotransplantation, retaining both multipotency and migratory capacity, but more remarkably, HNSC transplantation improved cognitive function in 24-month-old rats.

MATERIALS AND METHODS

Detailed methods for the maintenance and proliferation of HNSCs have been described previously [4]. Briefly, the HNSCs were cultured in 20 ml serum-free supplemented growth medium consisting of HAMS-F12 (Gibco, BRL, Burlington, ON); antibiotic–antimycotic mixture (Gibco); B27 (Gibco); human recombinant FGF-2 and EGF (R and D Systems, Minneapolis, MN) and heparin (Sigma, St. Louis, MO). The cells were incubated at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA). To facilitate optimal growth conditions, HNP spheroids were sectioned into quarters every 2 weeks and fed by replacing 50% of the medium every 4–5 days.

Matured (6 months old) and aged (24 months old) male Fischer 344 rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Using bregma as a reference point, about 10^5 cells were collected and slowly injected into the right lateral ventricle (AP −1.4; ML 1.8; DV 3.8 mm) of the brain using a stereotaxic apparatus (Devid Kopff). Immunosuppressant was not given to the animals. The memory score was tested before and after the injection of cells using the Morris water maze.

All animal experiments were conducted in strict accordance to guidelines of the university animal care committee. The Morris water maze was conducted as described before [5]. The water maze consisted of a large, circular tank (diameter 183 cm; wall height 58 cm) filled with water (27°C) opacified by the addition of powdered milk (0.9 kg). Beneath the water surface (1 cm), a clear escape platform (height, 34.5 cm) was positioned near the center of one of the four quadrants of the maze. The rats received three training trials per day for 7 consecutive days, using a 60 s inter-trial interval. A training trial consisted of placing the animal in the water for 90 s or until it successfully located the platform. If the rat failed to find the platform within the 90 s it was gently guided to the platform. For spatial learning assessment, the platform's location remained constant in one quadrant of the maze, but the starting position for each trial was varied. Every sixth trial was a probe trial, during which the platform was retracted to the bottom of the pool for 30 s and then raised and made available for escape. The training trials assess the acquisition and day-

to-day retention of the spatial task while the probe tests are used to assess the search strategy. At the completion of the spatial learning assessment, one session with six trials of cue training was performed. Rats were trained to escape to a visible black platform raised 2 cm above the surface of the water. The location of the platform was varied from trial to trial in order to assess sensorimotor and motivational functioning independent of spatial learning ability. Each rat was given 30 s to reach the platform and allowed to remain there briefly before the 30 s inter-trial interval. Accuracy of performance was assessed using a learning index score computed from the probe trials. The learning index is a derived measure from an average proximity (cumulative search error divided by the length of the probe trial) on the second, third, and fourth interpolated probe trials. Scores from these trials were weighted and summed to provide an overall measure of spatial learning ability. Lower scores on the index indicate a more accurate search in the vicinity of the target location; higher scores indicate a more random search and poor learning.

At 30 days post-transplantation, the rats were sacrificed by an overdose of sodium pentobarbital (70 mg/kg, i.p.) and perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Brains were removed, placed into 4% paraformaldehyde fixative containing 20% sucrose and left overnight. The brains were sliced into 20 μm coronal sections using a cryomicrotome. The sections were washed briefly in PBS and pretreated with 1 M HCl for 30 min at room temperature and neutralized with sodium borate (0.1 M, pH 8.0) for 30 min in order to increase the accessibility of the anti-bromodeoxyuridine (BrdU) antibody to the BrdU incorporated in the cell nuclei. After rinsing with PBS, sections were transferred to the solution containing 0.25% Triton X-100 in PBS (PBST) for 30 min. Then the sections were blocked in PBST containing 3% donkey normal serum for 1 h and incubated with sheep anti-BrdU (1:1000; Jackson IR Laboratories, Inc. West Grove, PA) or mouse anti-BrdU (1:200; DSHB, Iowa City, IA) diluted in PBST overnight at 4°C. After rinsing in PBS, donkey anti-mouse or donkey anti-sheep conjugated to rhodamine IgG (Jackson IR Laboratories, Inc.) was added at a 1:200 dilution in PBST for 2 h at room temperature in the dark. Then the sections were washed with PBS and incubated with mouse IgG2b monoclonal anti-human βIII-tubulin, clone SDL3D10 (1:500, Sigma), goat anti-human-glial filament protein (GFAP), *N*-terminal human affinity purified (1:200, Research Diagnostics Inc., Flander, NJ) and Mouse IgG1 monoclonal anti-GFAP, clone G-A-5 (1:500, Sigma), respectively, overnight at 4°C in the dark. The corresponding secondary antibodies for them were donkey anti-mouse (1:200) and donkey anti-goat IgG (H+L; 1:200) conjugated to FITC (Jackson IR Laboratories, Inc.), respectively. Following a brief PBS washing, they were added into sections for 2 h incubation at room temperature in the dark. Sections were then washed thoroughly with PBS before mounting to glass glides. The mounted sections were covered with Vectashield with 4',6-diamidine-2-phenylindole.2HCl (DAPI, Vector Laboratories, Inc., Burlingame, CA) for fluorescent microscopic observation. Microscopic images were taken by using the Axiocam digital camera mounted on the Axioscope 2 with Axiovision software (Zeiss).

RESULTS

The HNSCs were expanded without differentiation under the influence of mitogenic factors in supplemented serum-free media [3]. To differentiate between host and transplanted cells, the nuclei of the HNSCs were labeled by the incorporation of BrdU into the DNA. These labeled cells were subsequently injected unilaterally into the lateral ventricle of matured (6-month-old) and aged (24-month-old) rats. The cognitive function of these animals was assessed by the Morris water maze [5] before and 4 weeks after the transplantation of HNSCs. Before the HNSCs transplantation, some of the aged animals (aged memory unimpaired animals) had cognitive function in the range of the matured animals, while others (aged memory impaired animals) had cognitive function entirely out of the range of the matured animals (Fig. 1a). After the HNSC transplantation, most of the aged animals had cognitive function in the range of the matured animals. Strikingly, one of the aged memory impaired animals displayed behavior that was dramatically better than the level of the matured animals (Fig. 1a). Statistical analysis showed that cognitive function significantly improved in both matured ($p < 0.001$, $n = 8$) and aged memory impaired animals ($p < 0.001$, $n = 6$). In contrast, no improvement in cognitive function was observed in vehicle injected control animals ($n = 6$), or aged memory unimpaired animals ($n = 7$) after the HNSC transplantation (Fig. 1b). Three of the 13 aged animals showed deterioration of performance in the water maze after the HNSC transplantation. This fact needs to be further analyzed, but this may be due to the deterioration of the physical strength of these animals during the experimental period.

To investigate the morphology and population of differentiated HNSCs, we further analyzed brain sections taken after the second water maze task by immunohistochemistry with cell specific markers. The transplanted HNSCs, with BrdU-immunopositive nuclei, were stained for human βIII-tubulin and human GFAP. Double immunolabeling with βIII-tubulin and BrdU in 3 different planes from the same microscopic view clearly shows the co-localization of these two signals in the same cells (Fig. 2). According to the manufacturer's description, the anti-βIII-tubulin antibody may recognize the host (rat) βIII-tubulin. Despite this, the specific co-localization of these βIII-tubulin and BrdU at different planes indicates that the majority of βIII-tubulin-immunopositive cells are indeed transplanted HNSCs. This may due to the fact that βIII-tubulin is mainly expressed in immature neurons, the majority of which are transplanted HNSCs in this study. The presence of these cell-specific antigens indicates that the transplanted HNSCs successfully differentiated into neurons and astrocytes, respectively. Immunohistochemical analysis of brain sections revealed cells intensely and extensively positive for human βIII-tubulin staining. Specifically, these cells were located primarily in the bilateral cingular and parietal cortexes (layer II, IV and V; Fig. 3a,b) and hippocampus (CA1, dentate gyrus and CA2; Fig. 3c–e).

The transplanted HNSCs also differentiated into GFAP-immunopositive staining cells localized near the area where neuronal cells were found. Further analysis with double immunostaining revealed that donor-derived astrocytes co-localized with the neuronal fibers in the cortex

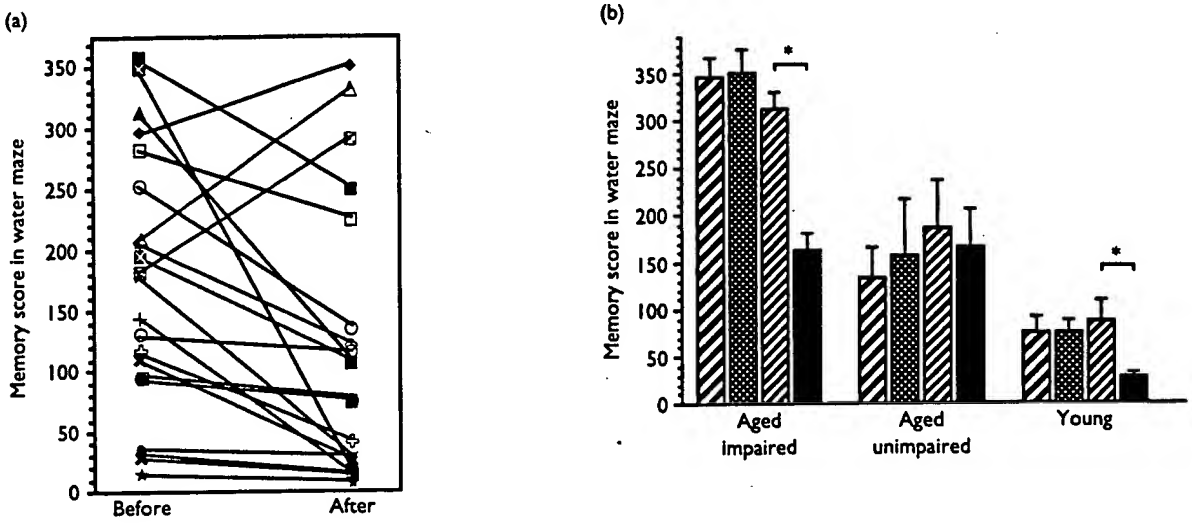


Fig. 1. Effect of HNSC transplantation on memory score in the water maze. (a) Individual memory score before and after the transplantation shows improvement in the majority of the animals. ■: Aged memory impaired animals, ▩: Aged memory unimpaired animals, ■: Matured animals. (b) Mean of memory score in each animal group before (▨) and after (▩) HNSC transplantation shows a significant improvement in aged memory impaired and young animals. The animals that received vehicle injection do not show significant difference in memory scores between before (▨) and after (■) the injection.

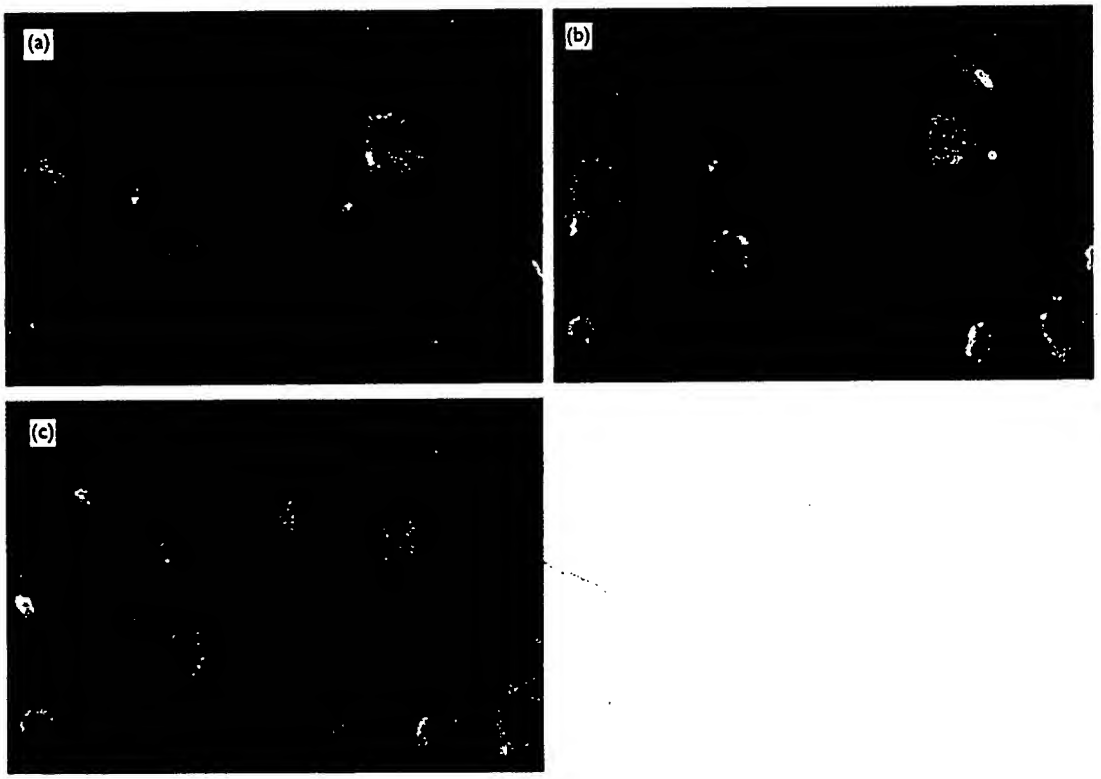


Fig. 2. Co-localization of βIII-tubulin and BrdU immunoreactivity in the same cells. (a–c) Three different planes of the same microscopic view. The βIII-tubulin-positive cells (green) show BrdU-positive nuclei (red) indicating that these cells are derived from transplanted HNSCs.

layer III (Fig. 3f) and CA2 region of hippocampus (Fig. 3e). These donor-derived astrocytes were large compared with host glia, having cell bodies 8–10 μm in diameter with thick processes and BrdU-immunopositive nuclei (Fig. 3g). We did not detect the above-mentioned morphologies and distribution of GFAP positive cells in the control rats that received no HNSC transplantation. When we stained with an anti-GFAP antibody that recognizes rat GFAP (Sigma,

clone G-A-5), the host astrocytes had small cell bodies with multiple delicate processes, which were distributed mainly in the white matter and around the edge of the brain (data not shown).

DISCUSSION

There are two possible mechanisms to explain the beneficial effects of the HNSC transplantation on cognitive function of the host brain. One is replacement or augmentation of neuronal circuit by the HNSC-derived neurons and other is the neurotrophic action of factors released from the transplanted HNSCs. Although the following morphological study shows extensive incorporation of HNSCs and massive growth of neuronal fibers in the host brain area related to spatial memory task, HNSCs may still migrate toward the damaged neurons and rescue them by the production of neurotrophic factors. Therefore, a synergism between these two mechanisms may exist and allow for the successful transplantation.

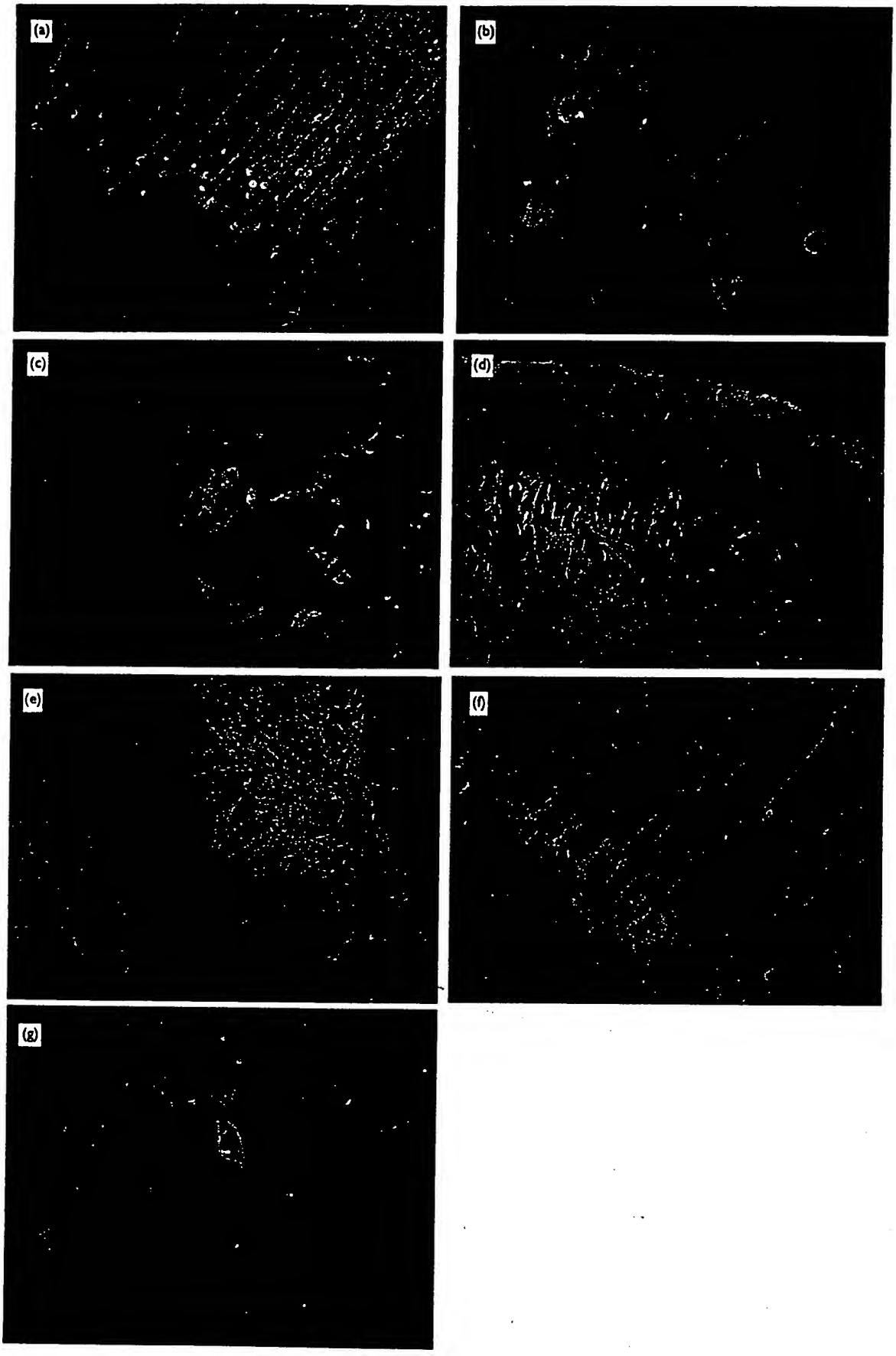
Since the spatial memory of HNSC-transplanted animals as assessed by the Morris water maze improved, incorporation of HNSCs into the brain areas known to be related to spatial memory [5,6] allowed for an improvement in spatial memory. Although βIII-tubulin is considered to be an early neuronal marker and physiological and electromicroscopic investigations will be required to determine the functional incorporation of these HNSC-derived neurons, the morphological observation indicates that functional association of these cells to the host brain occurred. Further histochemical analysis revealed that the βIII-tubulin-positive donor-derived cells found in the cerebral cortex were characterized by having dendrites pointing to the edge of the cortex whereas in the hippocampus, donor-derived neurons exhibited morphologies with multiple processes and branches. These differential morphologies of the transplanted HNSCs in different brain regions indicate that site-specific differentiation of HNSCs occurs according to various factors expressed in each brain region.

We observed strong astrocyte staining in the frontal cortex layer 3 and CA2 region of hippocampus, areas where astrocytes are not normally present in the animal. The migration of HNSCs to the CA2 raises particular interest because CA2 pyramidal neurons highly express bFGF and the expression of bFGF is up-regulated by entorhinal cortex lesions [7–9]. The CA2 pyramidal neurons in the host brain may express bFGF as a response to a reduction of synaptic transmission, an event that may occur during aging. Subsequently, this expressed bFGF can act as a signal for the transplanted HNSCs to respond, migrate and/or proliferate under the influence of bFGF produced in the host brain after the transplantation.

The regions rich in astrocyte staining are also the same regions where the extensively stained neuronal fibers were identified (Fig. 3a,c,e). During development glial cells have many complex functions, such as neuronal and axonal guidance, and production of trophic factors [10]. It has been suggested that following transplantation, migrating glial cells guide and support the growth and extension of neuronal fibers [11]. However, other studies have argued that glial cells may be detrimental by forming an extensive interface between the host and graft [12]. Although the mechanism(s) of glia–neuron interaction in the HNSC-transplanted host brain is not well understood, this overlapping distribution of glial and neuronal fibers strongly suggests that this interaction plays a pivotal role in the survival, migration, and differentiation of transplanted HNSCs.

The most significant difference in our experimental procedure is the lateral intraventricular transplantation of undifferentiated HNSCs in the form of neuro-spheroids. While many studies were done with intra-tissue injection of dissociated and partially differentiated NSCs [4,13–15], we employed spheroid injection because the dissociation of neuro-spheroids is known to cause immediate senescence of NSCs and increase the vulnerability of NSCs in culture [16]. Another added benefit of intraventricular injection is that since there is less tissue destruction, it may induce less recruitment of immune cells by the host. This is evidenced by the lack of increased host astrocyte staining without any immunosuppression. The mechanism behind transplanted cell migration into the brain through the ventricle is as yet still unknown. However, our results indicate that the mechanism may lie behind a direct integration into the host brain. Specifically, immunohistochemical analysis revealed that some of the BrdU cells were found to be situated along the lateral ventricular wall while a few appeared to have integrated directly into the cells lining the ventricle. Similar observations were reported in a variety of studies using neuronal transplantation to the lateral ventricle of animals. The intraventricular transplantation used in this study may provide an alternative route to the site-specific injection by which the grafted cells may gain access to various structures by the flow of CSF.

Fig. 3. Typical fluorescent immunohistochemical pictures in the aged rat brain 30 days after HNSC transplantation. We used βIII-tubulin and GFAP immunoreactivity as markers for neuron and glia, respectively. **(a)** HNSCs migrated into the cortex and differentiated into neurons as indicated by the βIII-tubulin-positive cells (green), which have morphologies typical of pyramidal cells in layer IV and V of the parietal cortex. Apical dendrites were pointed towards to the edge of the cortex. Since we labeled HNSC DNA with BrdU before the transplantation, the transplanted cells will have BrdU-positive nuclei (red). Contrarily, the host cell's nuclei are counterstained with DAPI (blue). Many cells with BrdU-positive nuclei are observed with βIII-tubulin immunoreactivity in layer II and without βIII-tubulin immunoreactivity in the layer III. **(b)** Higher magnification of the parietal cortex in cortex layer IV: All the βIII-tubulin-immunoreactive (green) positive cells show BrdU-positive nuclei (red) while many other host cell's nuclei are stained with only DAPI (blue). HNSCs tend to have larger nuclei than host cells. **(c)** HNSCs migrated into the hippocampus and differentiated into βIII-tubulin positive cells (green), having morphologies typical of pyramidal cells in CA1 pyramidal cell layer. These βIII-tubulin-positive cells have BrdU-positive-nuclei (red), indicating that these cells originated from transplanted cells. In contrast, host cell nuclei counterstained with DAPI (blue) are not βIII-tubulin-positive. **(d)** Hippocampus CA2 shows a large number of GFAP-positive cells (red) and βIII-tubulin-positive cells (green). **(e)** In the dentate gyrus-as well as the βIII-tubulin-positive cells (green) and GFAP-positive cells (red), many fibers were βIII-tubulin positive. **(f)** βIII-tubulin-positive cells (green) and GFAP-positive cells (red) were found in layer IV and layer III, respectively. We have not seen such a layer of astrocytes in normal rats without HNSC transplantation. **(g)** These GFAP-positive cells (green) show BrdU-positive nuclei (red), and have larger cell bodies and thicker processes than the host (rat) astrocytes.


(a)
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Following immunohistochemistry, a symmetrical distribution of neurons and astrocytes at both sides of the host brain was observed, indicating that the progeny of these HNSCs have a great potential for migration. Although astrocytes have been shown to migrate over long distances following transplantation [17–19], there is experimental evidence showing that neurons do not migrate as widely as glial cells [20]. In our study, the neuronal precursors derived from the HNSCs seem to possess similar migratory ability as the astrocyte precursors. This may be due to the fact that we transplanted undifferentiated HNSCs and such an immature stage for both glia and neuron possesses the potential to migrate over long distances. The extensive incorporation of neuronal and glial cells found in the cortex and other sub-regions of the hippocampus may be interpreted as evidence for the significance of local cues or signals within these regions which enable these grafted NSCs to migrate. It remains to be demonstrated, however, to what extent these newly formed neurons can undergo complete maturation with physiologically functional connections to the host brain.

Many studies have discovered the existence of endogenous precursor cells in certain regions of the brain. These regions include the anterior subventricular zone (SVZ) and the hippocampal dentate gyrus, areas where neurogenesis continues into adulthood in mammalian species, including humans [21–23]. The presence of multipotent neural cells in the adult brain similar to the fetal neural stem cell in these brain regions indicates the importance of microenvironments to neural progenitors. Aging is characterized by increased levels of inflammation in the CNS [24,25]. Thus, it is reasonable to speculate that factors existing in the environment of the aged brain may direct the non-neuronal differentiation pathway. However, in our current study, the transplanted HNSCs successfully generated many morphologically functional neurons in the aged animals. In a previous study, we observed that initial glial differentiation of HNSCs was followed by neuronal differentiation in a serum-free culture media without any additional factor [3]. This finding suggests that glial differentiation caused by the serum deprivation produced factors that allowed neurons to differentiate. Since we observed an association of astrocytes and neurons derived from HNSCs in this study, we may have to consider the possibility that the donor astrocytes may direct the neuronal differentiation.

CONCLUSION

In order to facilitate therapeutic HNSC application to the general adverse consequences of aging and neurodegenerative diseases, it is important to understand these environmental factors which direct the differentiation fate of these HNSCs to diverse lineages *in vivo*. While future studies are needed to elucidate these environmental factors, we have none the less demonstrated that HNSC transplantation into the brains of aged memory impaired rats significantly improved their cognitive function. Moreover, not only did the HNSCs successfully differentiate into neurons and astrocytes, but more importantly, both neurons and astrocytes migrated to the cortex and hippocampus in a well-defined and organized pattern in the adult brain.

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Transplantation of Clonal Neural Precursor Cells Derived from Adult Human Brain Establishes Functional Peripheral Myelin in the Rat Spinal Cord

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We examined the myelin repair potential of transplanted neural precursor cells derived from the adult human brain from tissue removed during surgery. Sections of removed brain indicated that nestin-positive cells were found predominately in the subventricular zone around the anterior horns of the lateral ventricle and in the dentate nucleus. Neurospheres were established and the nestin-positive cells were clonally expanded in EGF and bFGF. Upon mitogen withdrawal *in vitro,* the cells differentiated into neuron- and glia-like cells as distinguished by antigenic profiles; the majority of cells in culture showed neuronal and astrocytic properties with a small number of cells showing properties of oligodendrocytes and Schwann cells. When transplanted into the demyelinated adult rat spinal cord immediately upon mitogen withdrawal, the cells elicited extensive remyelination with a peripheral myelin pattern similar to Schwann cell myelination characterized by large cytoplasmic and nuclear regions, a basement membrane, and P0 immunoreactivity. The remyelinated axons conducted impulses at near normal conduction velocities. This suggests that a common neural progenitor cell for CNS and PNS previously described for embryonic neuroepithelial cells may be present in the adult human brain and that transplantation of these cells into the demyelinated spinal cord results in functional remyelination.

Key Words: **neural precursor cells; axon; demyelination; glia; myelin; multiple sclerosis; Schwann cell.**

INTRODUCTION

Multipotent precursor or stem cells are present in the mammalian central nervous system (CNS) during development and in the adult brain (16, 26, 33, 38, 42, 48). A recent study has demonstrated that neurospheres can be developed from multipotent/progenitor cells from neurogenic regions of the adult human brain (30). Neural precursor cells can be isolated and expanded in culture in the presence of mitogens such as epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) (8, 17, 20, 27, 53). After withdrawal of the mitogens and with appropriate growth factors or substrates these cells can differentiate into neurons or glia (44, 48). When transplanted into the embryonic or neonatal CNS both neurons (6, 50, 53) and oligodendrocytes (20, 37) have been generated. These cells appear to differentiate and integrate into the host CNS because they form functional synapses (neurons) and myelinate (oligodendroctyes) axons. However, when injected into the adult CNS, stem cells differentiate into primarily astrocytes (35). These results indicate that environmental signals may direct the specification of cell lineage.

Multipotential neural progenitor cells derived from the fetal human brain propagate and differentiate in culture and *in vivo* (10, 39, 52). Progenitor cells from adult animals have been cultured from the subependymal zone (SEZ) (25, 26, 38, 48), the subventricular zone (SVZ) (33, 38), the hippocampus (16, 42), and the spinal cord (27, 40, 46). A recent study suggested that ependymal cells may be a source of progenitor cells (25), but a GFAP-positive cell distinct from, but adjacent to, ependymal cells has been recently implicated as the primary neural progenitor cell type of the subventricular region (13).

While oligodendrocytes normally myelinate CNS axons, Schwann cells can remyelinate CNS axons after injury (14) and following transplantation into the demyelinated CNS (4, 22). Schwann cells can be derived from single cell clones of neural crest cells (31). Mujtaba *et al.* (40) have distinguished a common neural progenitor for the PNS and the CNS. They found that cultured neuroepithelial cells derived from embryonic rat spinal cords can differentiate into CNS precursors

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TABLE 1

Tissue Derivation Sites for Cultures from Patients with Lobe Resections for Tumor Removal

Case	Age/sex	Diagnosis	Location	Culture
1	35/F	Glioma	Frontal lobe (SVZ/SEZ)	+
2	19/M	Glioblastoma	Frontal lobe (SVZ/SEZ)	+
3	64/F	Glioblastoma	Temporal cortex	−
4	62/F	Glioma (low grade)	Temporal lobe, hippocampus	+
5	44/F	Glioma (low grade)	Frontal cortex	−

Note. The patients ranged in age from 19 to 64 years old and all had a diagnosis of glioma or glioblastoma. Neural progenitor cells were cultured from frontal and temporal lobe tissue which included periventricular, subependymal, or subventricular zones. Cultures derived from frontal or temporal cortex did not yield neural progenitor cells in culture.

which can differentiate into CNS neurons and glia and PNS precursors which can differentiate into neural crest cells which give rise to peripheral neurons, Schwann cells, and smooth muscle. Recently, Keirstead *et al.* (28) demonstrated that immunoselected precursor cells from neonatal rat forebrain expressing the polysialyated (PSA) form of the neural cell adhesion molecule (NCAM), which mostly generate oligodendrocytes and astrocytes *in vitro,* can produce peripheral myelin *in vivo.*

To test the ability of neural precursor cells derived from the adult brain to differentiate into myelin-forming cells and repair the adult demyelinated CNS, we transplanted clonal neural progenitor cells derived from the adult human brain into an experimentally established glial-free zone in the dorsal columns of the rat spinal cord. Although these precursor cells differentiated upon mitogen withdrawal in culture into neurons and astrocytes and to a lesser extent oligodendrocytes, when transplanted into a demyelinated glial-free zone of the adult rat spinal cord, they extensively remyelinated the axons and restored near normal conduction velocity. The majority of the myelinated axons displayed a peripheral pattern of myelination which is characterized by P0 immunoreactivity, large nuclear and cytoplasmic regions of the myelin-forming cells surrounding the axons and a basement membrane. These data provide evidence that clonal neural precursor cells derived from the adult brain can give rise to Schwann-like cells which form functional myelin when transplanted into an axon-enriched glial-free environment of adult central white matter. Thus, these data suggest that a common neural progenitor cell for the CNS and the PNS described for embryonic neuroepithelial cells (40) may also be present in the adult human brain.

METHODS

Derivation of Adult Human Tissue

Brain tissue was obtained from five patients undergoing lobe resection for tumor (glioma) removal (Table 1). Either tissue was fixed and prepared for sectioning and immunohistochemistry or selective regions were dissociated for preparation of neurospheres. As our cells were derived from the adult human brain removed because of glioma, criteria were established to distinguish the neurospheres from the glioma cells. First, when cultured alone the glioma cells did not float but adhered to the bottom of the culture flasks. Moreover, they continued to propagate even when they became confluent; the neurosphere-derived cells stopped dividing upon becoming confluent. We also removed tissue from regions remote from the site of the glioma. The glioma cells continued to propagate with and without the presence of mitogens. The proliferative properties of the glioma cells, their adherence to the bottom of the flasks, and their inability to produce small floating cells that coalesce to form neurospheres indicate that these cells were likely not present in the neurosphere cultures utilized for this study.

Nestin Immunoreactivity in Brain Slices

The whole human brain was obtained from a cadaver of a 24-year-old female. The cerebral cortex, subventricular zone, and hippocampus were fixed with 4% paraformaldehyde in 0.14 M Sorensen's phosphate buffer (pH 7.4) at 4°C for 24 h and dehydrated with 30% sucrose in 0.1 M phosphate-buffered saline (PBS) for overnight. The tissues were then placed in OCT compound (Miles Inc.) and frozen in liquid N_2, and 10-μm sections were cut with a cryostat. Sections were dried onto silane-coated slides. Immunohistochemistry was carried out using an anti-nestin antibody (nestin; 1:5000 anti-monoclonal mouse anti-nestin, Chemicon). The primary antibody was visualized using Vectastain ABC-AP mouse IgG kit (Vector Laboratories) and alkaline phosphatase substrate kit 4 (BCIP/NBT; Vector Laboratories) according to the manufacturer's instruction. After immunostaining, slides were covered by coverglasses using Crystal/Mount (Biomeda Corp.). Photographs were taken on a Zeiss microscope (Axioskop FS).

Primary Culture of Adult Human Neural Precursor Cells

Tissue samples were obtained from frontal cortex, temporal cortex, hippocampus, and the subventricular/subependymal zone of the frontal lobe in adult humans operated on to remove brain tumors (summarized in Table 1). The samples were dissected in L-15 medium; rinsed; enzymatically treated in L-15 containing 0.01% DNase I, 0.25% trypsin, and 0.1% collagenase at 37°C for 30 min; and mechanically dissociated by brief trituration with a fire-polished silicon-coated Pasteur pipette. The cells were collected by centrifugation, resuspended in serum-free medium (NPMM, neural progenitor cell maintenance medium, Clonetics, San Diego, CA) supplemented with 10 ng/ml bFGF and 10 ng/ml EGF, and plated onto 100-mm^2 laminin-coated tissue culture plates at 8×10^5 cells per plate. The next day, the cells were resuspended and then plated onto 100-mm^2 noncoated culture plates. Six hours later, the supernatant was collected and replated onto 100-mm^2 noncoated culture plates. Cells were maintained at 37°C in 5% CO_2/95% O_2. bFGF and EGF were added daily and culture medium was changed weekly. Spherical masses (i.e., neurospheres) became visible after 7–10 days in culture.

Clonal Expansion and Induced Differentiation of Adult Human Neural Precursor Cells

A spherical mass of cells in the primary culture dish was collected under microscopy and was dissociated by incubation in HBSS containing 0.05% trypsin and 0.01% DNase I. The dissociated cells were cloned by limiting dilution in 96-well plates. After the single cell expansion, a spherical mass of cells in the secondary culture was collected and the same procedure was repeated for further subcloning. In all processes, cells which were not dissociated well were discarded to avoid contamination.

Differentiation of the clones was initiated by enzymatically and mechanically dissociating the cellular sphere (neurosphere) and culturing on polyethyleneimine-pretreated plates in the absence of mitogen.

Phenotypic Analysis in Vitro: Immunocytochemistry

Cultured cells were rinsed in PBS and fixed for 15 min with a fixative solution containing 4% paraformaldehyde in 0.14 M Sorensen's phosphate buffer, pH 7.4, 4°C. Fixed cells were incubated for 15 min in a blocking solution containing 0.2% Triton X-100 and 5% normal goat serum before incubation with the primary antibody. The primary antibodies used were anti-α-microtubule-associated protein 2 (MAP-2; 1:10,000 monoclonal mouse anti-MAP-2, Upstate Biotechnology), anti-τ (τ 1:1,000 monoclonal mouse anti-τ, Sigma), anti-β-tubulin type III (TUJ-1; 1:500 monoclonal mouse anti-TUJ-1, Babco), anti-neurofilament (NF; 1:1,000 monoclonal mouse anti-NF, Nitirei), anti-neuron-specific enolase (NSE; 1:1,000 polyclonal rabbit anti-NSE, Nitirei), anti-glial fibrillary acidic protein (GFAP; 1:200 polyclonal rabbit anti-GFAP, Nitirei), anti-O4 (O4; 1:100 monoclonal mouse anti-O4, Boehringer Mannheim), anti-galactocerebroside (GalC; 1:200 monoclonal mouse anti-GalC, Boeringer Mannheim), anti-nestin (nestin; 1:5,000 monoclonal mouse anti-nestin, Chemicon), anti-A2B5 (1:100 monoclonal mouse anti-A2B5, Boehringer Mannheim), anti-vimentin (Vim; 1:100 monoclonal mouse anti-Vim, Nitirei), anti-peripheral myelin protein (P0; 1.200 monoclonal rabbit anti-P0 antibody, kindly provided by Dr. D. Colman), and anti-S-100 protein (S-100; 1:1000 polyclonal rabbit anti-S-100, Nitirei). Triton-X was omitted in the reaction with A2B5, GalC, and O4 primary antibody. The primary antibody was visualized using goat anti-mouse and goat anti-rabbit IgG antibody with fluorescein (FITC) (1:100, Jackson ImmunoResearch Laboratories, Inc.) or alkaline phosphatase reaction (Zymed) according to the manufacturer's instructions. After immunostaining, coverslips were mounted cell side down on microscope slide using mounting medium (Dako). Photographs were taken on a Zeiss immunofluorescent microscope (Axioskop FS).

LacZ Transfection into the Clonal Adult Human Neural Precursors

An expression vector for mammalian cells which contains the LacZ gene was used to transduce the bacterial β-galactosidase (β-gal) gene into clonally expanded neural precursors derived from the human brain. Clones of neural precursors were transfected by pcDNA3.1/His/LacZ (Invitrogen) constructed by cloning the β-gal gene into the pcDNA vector. The CMV provided the promoter for the β-gal gene. The simian virus 40 early promoter and the neomycin resistance gene, transmitting G418 resistance, are present downstream from the β-gal gene to permit selection of transfected colonies. Lipofectamine (20 μg/ml; Gibco) was used to transfect the expression vector pcDNA3.1/His/LacZ (10 μg/ml) to cultured precursors, which were rapidly proliferating under the influence of mitogen. Transfected precursors were then selected by incubation with the neomycin analogue G418 (400 μg/ml). Five rats received transplants from neuroprecursors transfected with the LacZ gene.

Animal Preparation and Transplantation

Experiments were performed on 12-week-old Wistar rats (8 unoperated controls, 10 demyelinated, and 15 demyelinated with transplants). The transplant experiments ($n = 15$) were carried out in three groups of 5 rats for a repeat of three times. A focal demyelinated lesion was created in the dorsal column of the spinal

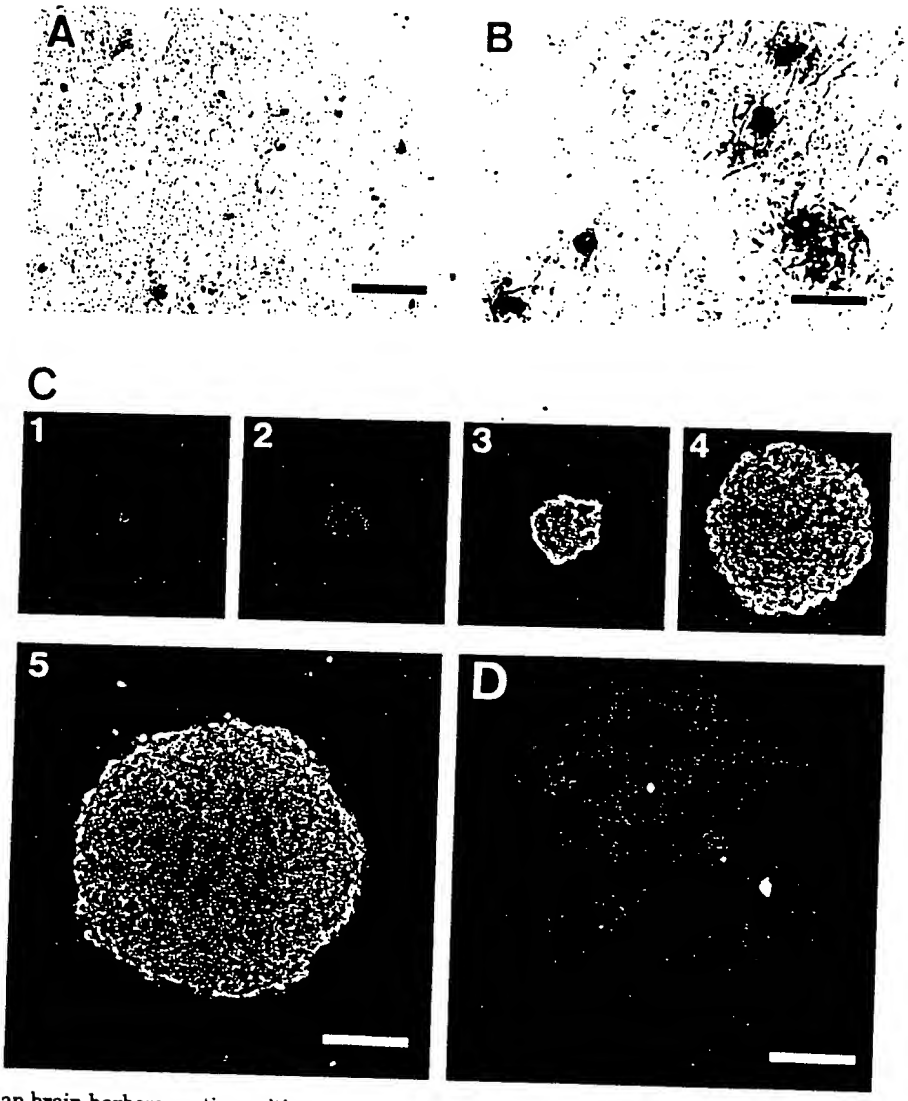

FIG. 1. The adult human brain harbors nestin-positive precursor cells. Immunohistochemical examination demonstrates scattered islands of the neural precursor cells in SVZ/SEZ (A and B). Nestin-positive cells are visualized by alkaline phosphatase reaction (A, lower magnification, bar 100 μm; B, higher magnification, bar 25 μm). (C) Clonal expansion of cells derived from adult human brain *in vitro*. High-power microscopic images in cultures. (C1) A single precursor cell in one well of a 96-well culture plate. (C2) In the presence of mitogen, a spherical cell mass begins to form after 4 days. The spherical appearance of adult human precursors after 1 week (C3), 2 weeks (C4), and 4 weeks (C5) *in vitro*. (D) Nestin-labeled neural aggregate derived from single cell expansion. Nestin positivity is visualized with fluorescein (FITC) (C1–C5, bar 100 μm; D, bar 40 μm).

cord using X-irradiation and ethidium bromide injection (EB-X) utilizing a method similar to that of Honmou *et al.* (22). Briefly, rats were anesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg) ip, and a 40-Gy surface dose of X-irradiation was delivered through a 2 × 4-cm opening in a lead shield (4 mm thick) to the spinal cord caudal to the 10th thoracic level (T-10) using a Softex M-150 WZ radiotherapy machine (100 kV, 1.15 mA, SSD 20 cm, dose rate 200 cGy/min). Three days after irradiation, rats were anesthetized as above and, using sterile technique, a laminectomy was performed at T-11. The demyelinating lesion was induced by the direct injection of EB into the dorsal column via a drawn glass micropipette. Injections of 0.5 μl of 0.3 mg/ml EB in saline were made at depths of 0.7 and 0.4 mm near the midline of the dorsal columns at three longitudinal sites separated by 2 mm. A suspension of clonal progenitors (1×10^4 cells/μl) in 1μl medium was injected into the middle of the EB-X-induced lesion 3 days after the EB injection. Transplant-receiving rats were immunosuppressed with cyclosporin A (10 mg/kg/day, sc, kindly provided by Novartis Pharma AG, Basel, Switzerland).

Histological Examination

The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, ip) and perfused through the heart, first with PBS and then with a fixative solution containing 2% glutaraldehyde and 2% paraformalde-

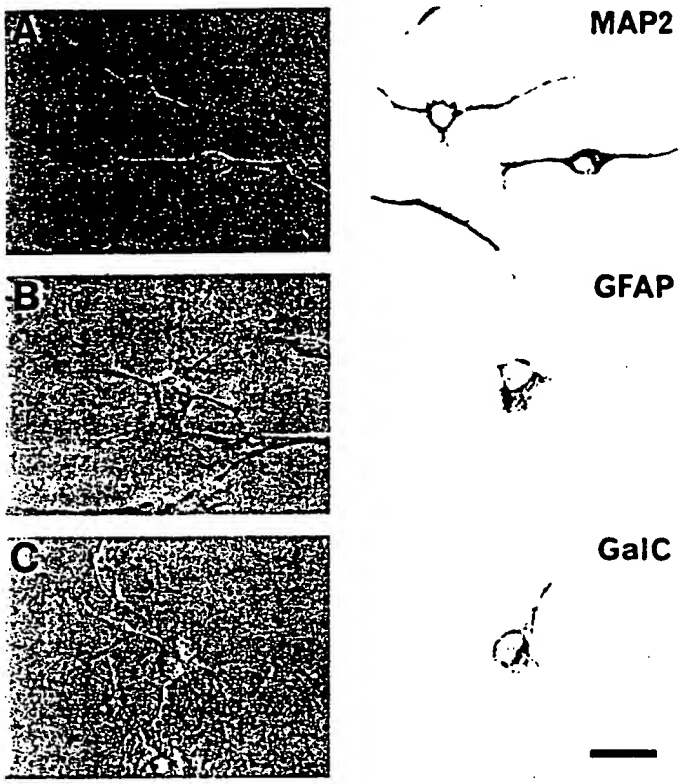

FIG. 2. Cell differentiation in culture after mitogen removal. Phase contrast photomicrographs showing a neuron-like cell (A, left), an astrocyte-like cell (B, left), and an oligodendrocyte-like cell (C, left). Immunolabeling of the cells with anti-MAP-2 (A, right), anti-GFAP (B, right), and anti-GalC (C, right) indicates neuronal, astrocytic, and oligodendrocytic phenotypes, respectively. The primary antibody was visualized using goat anti-mouse and goat anti-rabbit IgG antibody with alkaline phosphatase reaction. Bar, 25 μm.

hyde in 0.14 M Sorensen's phosphate buffer, pH 7.4. Following *in situ* fixation for 10 min the spinal cord was carefully excised, cut into 1-mm segments, and placed into fresh fixative. The tissue was washed several times in Sorensen's buffer, postfixed with 1% OsO_4 for 2 h at 25°C, dehydrated in graded ethanol solutions, passed through propylene oxide, and embedded in EPON. Thick sections (1 μm) were cut, counterstained with 0.5% methylene blue, 0.5% azure II in 0.5% borax, and examined with a light microscope (Zeiss: Axioskop FS). Semithin sections were counterstained with uranyl and lead salts and examined with a JEOL JEM1200EX electron microscope operating at 60 kV.

Detection of β-Galactosidase Reaction Products in Vitro and in Vivo

β-Gal-expressing cells were detected *in vitro* by incubating the cultured neurospheres with X-Gal to form a blue color within the cell (data not shown). Neurospheres were fixed in 0.05% glutaraldehyde, washed with PBS, and then incubated with X-Gal to a final concentration of 1 mg/ml in X-Gal developer (35 mM $K_3Fe(CN)_6$/35 mM $K_4Fe(CN)_6.3H_2O$/2 mM $MgCl_2$ in phosphate-buffered saline). Cells were then incubated at 37°C overnight and examined by light microscopy for the presence of a blue reaction product.

Three weeks after transplantation, β-galactosidase-expressing Schwann-like cells were detected *in vivo*. Spinal cords were removed and fixed in 0.5% glutaraldehyde in phosphate buffer for 1h. Sections (100 μm) were cut with a vibratome and β-galactosidase-expressing Schwann-like cells were detected by incubating the sections at 37°C overnight with X-Gal to a final concentration of 1 mg/ml in X-Gal developer to form a blue color within the cell. The slices were then fixed in 10% paraformaldehyde in phosphate buffer overnight, dehydrated, and embedded in paraffin. Transverse sections (3 μm) were cut and examined by light microscopy (Zeiss; Axioskop FS) for the presence of a blue reaction product (β-galactosidase reaction product).

Phenotypic Analysis in Vivo: Immunohistochemistry

Three weeks after transplantation, the peripheral myelin protein P0-expressing myelin-forming cells were detected *in vivo*. The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, ip) and perfused through the heart, first with PBS and then with a fixative solution containing 10% paraformaldehyde in 0.14 M Sorensen's phosphate buffer, pH 7.4. Spinal cords were removed, fixed in 10% paraformaldehyde in phosphate buffer for overnight, dehydrated, and embedded in paraffin. Transverse sections (3 μm) were cut. Paraffin wax-embedded sections were dewaxed in xylene and treated with 1% hydrogen peroxide. Monoclonal rabbit anti-P0 antibody (1:200), polyclonal rabbit anti-NSE antibody (1:1000), and polyclonal rabbit anti-GFAP antibody (1:200) were applied. The primary antibody was visualized using goat anti-rabbit IgG antibody with peroxidase reaction. The nucleus was counterstained with hematoxylin. After dehydration with 70% alcohol, coverslips were mounted tissue side down on microscope slide using mounting medium (Dako). Photographs were taken on a Zeiss microscope (Axioskop FS).

Field Potential Recording

After induction of deep anesthesia (sodium pentobarbital 50 mg/kg, ip), the spinal cords of control (n = 5), demyelinated (n = 5), and transplanted rats (n = 5) were quickly removed and maintained in an *in vitro* submersion-type recording chamber with a modified Krebs' solution (containing 124 mM NaCl, 26 mM $NaHCO_3$, 3mM KCl, 1.3 mM NaH_2PO_4, 2 mM $MgCl_2$, 10 mM dextrose, 2 mM $CaCl_2$; saturated with 95% O_2 and 5% CO_2) (Fig. 6A). Field potential recordings of compound action potentials were obtained with glass microelectrodes (1–5 MΩ; 1 M NaCl) positioned in the dorsal columns, and signals were amplified with a

high-input impedance amplifier (Axoclamp 2A; Axon Inc.) and stored on a digitizer (Nicolet Pro 34). The axons were activated by electrical stimulation of the dorsal columns with bipolar Teflon-coated stainless-steel electrodes cut flush and placed lightly on the dorsal surface of the spinal cord. Constant current stimulation pulses were delivered through stimulus isolation units and the timing of the pulses was controlled by a digital timing device. The recorded field potentials were positive–negative–positive waves corresponding to source–sink–source currents associated with propagating axonal action potentials (22, 29); the negativity represents inward current associated with the depolarizing phase of the action potential.

All variances represent standard error (±SEM). Differences among groups were assessed by unpaired two-tailed t test to identify individual group differences. Differences were deemed statistically significant at $P < 0.05$.

RESULTS

Regional Distribution of Nestin-Positive Cells in the Adult Human Brain

Nestin immunoreactivity was studied in human brain sections obtained from the periventricular region of the frontal lobe, the hippocampal complex, and the frontal cortex. Islands of nestin-positive cells were found in each of these regions. The SEZ/SVZ regions located below the ependyma of the lateral margin of the anterior horn of the lateral ventricle contained a relatively high density of cells (Figs. 1A and 1B). The nestin-positive cells were either dispersed or localized in small groups. Within the hippocampal complex, the external surface of the dentate gyrus also contained a relatively high density of nestin-positive cells; Ammons horn (CA1–CA4) had a paucity of nestin-positive cells. Although frontal cortex had recognizable nestin-positive cells, they were scattered and much less dense and localized compared to the SEZ/SVZ and the dentate gyrus.

Brain tissue was removed from five patients (See Methods and Table 1) and divided from each patient for preparation of neurospheres in culture. We could prepare neurospheres from the SEZ/SVZ in two and from the temporal lobe/hippocampus in one patient (Table 1). We were unsuccessful in obtaining neurospheres from tissue in the temporal cortex and frontal cortex in two other patients. Neurospheres used in this study were prepared from the SEZ/SVZ from two patients.

Clonal Expansion of Nestin-Positive Cells

Nestin-positive cells isolated from the adult human brains were expanded by daily addition of EGF and bFGF in serum-free medium (see Methods). These cells grew as neurospheres and were expanded for a week or more in culture. A continuous supply of mitogens (EGF and bFGF) was important to repress differentiation and maintain a homogeneous population of self-renewing nestin-positive cells. As described below, upon mitogen withdrawal putative neuronal and glial lineages could be differentiated from these cells. In order to determine if the nestin-positive cells were generated by separate committed precursors or by a common multipotential precursor cell, a single cell clonal expansion method was used prior to mitogen withdrawal. Using the limiting dilution method (see Methods), individual dissociated cells (Fig. 1C1) from a sphere of nestin-positive cells were plated in a 96-well culture dish. An example of reestablishment of a neurosphere of nestin-positive cells from an individual cell is shown in Fig. 1C. Note the cellular proliferation in Figs. 1C1–1C5 over 4 weeks in culture. Figure 1D shows that these cells were indeed nestin positive after expansion. All expanded colonies displayed similar properties, thus indicating the clonal nature of the cells. Continued proliferation was observed for over 8 months *in vitro* in the presence of mitogen, and subclones could be established from these clonal cell lines allowing further expansion of the cells by repeating the limiting dilution method.

Characterization of the Human Precursor Cells Following Withdrawal of Mitogens in Culture

While the purpose of this study was not to study in detail lineage of the precursor cells but to study their fate when transplanted into a demyelinated region *in vivo*, we carried out some phenotypic characterizations to define our precursor cell population. To examine the multipotentiality of clones, expanded spheres of the nestin-positive clonal cells were dissociated and plated on polyethyleneimine-coated coverslips and maintained in culture in the absence of mitogen. At least three morphologically distinct cell types were observed from a dissociated neurosphere of clonally expanded cells (Figs. 2A–2C). The antigenic and morphological features of these cells were similar to those of rat stem cell cultures described in detail elsewhere (26). The three most common morphologies of cells were relatively small fusiform cells typically with two or three neurites (Fig. 2A), a larger multipolar cell (Fig. 2B), and a small spherical multipolar cell (Fig. 2C). Cells showing these morphologies stained positively for MAP-2, GFAP, and GalC, respectively (Figs. 2A–2C, right panels; different cells from the left panels), thus suggesting neuron-, astrocyte-, and oligodendrocyte-like differentiation.

The relative distribution of these cell types with various markers for neurons and glia is shown in Fig. 3. Note that the largest proportions of cells stained with MAP-2, TUJ-1, NSE, GFAP, and Vim. A2B5-labeled

cells were very limited. A very small proportion of cells were labeled by GalC and S100; O4 and P0 staining was virtually absent. This pattern of staining was similar from clone to clone. In a limited number of experiments dual immunolabeling was carried out to directly show multiple cell lineage derived from a clonal cell (data not shown); i.e., some cells stained positive for MAP-2 and TUJ-1 suggesting neuronal elements while others in the field were negative for those markers but positive for GFAP. We are careful with this level of analysis not to define these cells as being fully committed to neurons or astrocytes, but rather that upon mitogen withdrawal in culture that they differentiate in a pattern consistent with these phenotypes. These results are in agreement with other studies showing a relatively large number of neuron-like and astrocyte-like cells differentiating from neural precursor cells in culture after mitogen withdrawal, and a paucity of oligodendrocytes and Schwann cells (26, 27).

Transplantation of Neural Precursor Cells into a Glial-Free Spinal Cord Tract

The dorsal columns of the lumbar spinal cords were X-irradiated and subsequently injected with a nuclear chelator, ethidium bromide, to kill glial cells and to inhibit mitosis of endogenous glial cells (EB-X model; see Methods). The lesion induced by this procedure is characterized by virtually complete loss of endogenous glial elements (astrocytes and oligodendrocytes) with preservation of axons, i.e., a demyelinated lesion with no glia. The lesion occupies the entire dorsoventral extent of the dorsal columns for 5–7 mm longitudinally (5, 22, 23). No endogenous invasion of Schwann cells, oligodendrocytes, or astrocytes occurs before 6 to 8 weeks at which time these cells begin to invade the lesion from its peripheral borders. Thus, a demyelinated and glial-free environment *in vivo* is present for at least 6 weeks.

Myelinated axons in the normal dorsal columns are shown in the photomicrograph in Fig. 4A. After induction of an EB-X lesion virtually all of the axons are demyelinated, and astrocytes and oligodendrocytes are killed providing an aglial environment with preserved demyelinated axons and macrophages with cellular debris (Fig. 4B). Three weeks after injection of clonal human neural precursor cells into the central region of the lesion in immunosuppressed rats (cyclosporin A; see Methods), there was extensive remyelination of the axons (Figs. 4C and 4D). Remyelination was observed across the entire coronal dimension of the dorsal columns and considerably throughout the anteroposterior extent of the lesion. While it is well established that no endogenous remyelination by oligodendrocytes or Schwann cells occurs in this lesion model for at least 6 weeks (5), some donor cells were transfected with the reporter gene LacZ and X-Gal-positive cells were observed forming myelin (Fig. 4E).

The anatomical pattern of myelination was similar to that produced by Schwann cells, i.e., large cytoplasmic and nuclear regions surrounding the remyelinated axons (Fig. 4D, arrows). Immunoreactivity for the peripheral myelin-specific protein, P0, was observed in the myelin of the transplanted regions further indicating that Schwann cells were differentiated *in vivo* from the neural precursor cells (Fig. 4F). Electron microscopic examination of the remyelinated axons reveals ultrastructural features of peripheral myelin (Fig. 5A). The axons were ensheathed by relatively thick myelin surrounded by large cytoplasmic and nuclear regions characteristic of Schwann cell myelination (4, 22). Normal and demyelinated dorsal column axons are shown in Figs. 5B and 5C, respectively, for comparison. Moreover, a basement membrane, which is not observed around axons myelinated by oligodendrocytes, was observed around the myelin-forming cells after neural precursor cell transplantation (Fig. 5A, arrowheads). The morphological features and presence of P0 immunoreactivity indicate that the CNS-derived precursor cells differentiate *in vivo* into a cell with peripheral Schwann cell characteristics.

Restoration of Normal Conduction Velocity in the Remyelinated Axons

Spinal cords from control, demyelinated, and transplanted rats were removed and maintained in an *in vitro* recording chamber (see Methods). The dorsal columns were stimulated on the surface with bipolar electrodes and glass microelectrodes were used to record field potentials at various points through the lesion area (Fig. 6A). The recordings in Fig. 6B are superimposed traces of compound action potentials recorded 1

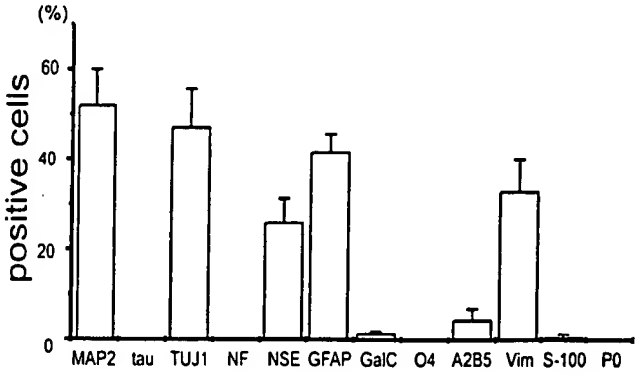

FIG. 3. Clones were differentiated in culture for 10 days in the absence of EGF and bFGF. Phenotypic analysis indicated a large proportion of cells expressing MAP-2, TUJ-1, NSE, GFAP, and Vim and a lesser proportion expressing GalC, A2B5, and S-100. Little expression of τ, NF, O4, and P0 was observed. This suggests that these immature cells had characteristics of neuron and astrocytes and to a lesser extent immature oligodendroctyes and Schwann cells.

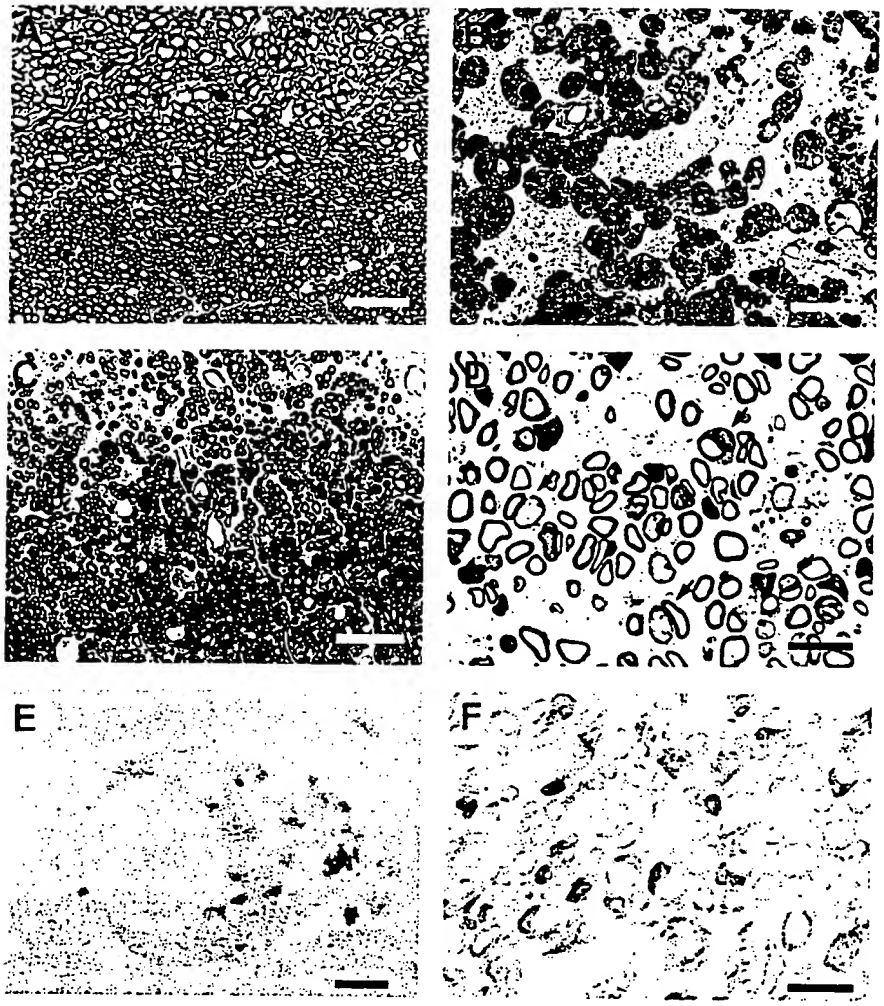

FIG. 4. Remyelination of the rat spinal cord following transplantation of adult human precursor cells. Normal (A), demyelinated (B), and remyelinated axons (C) of the dorsal column. (D) Remyelinated axons at higher magnification. The anatomical pattern of myelination was similar to that produced by Schwann cells (arrows). (E) The human cells in the rat EB-X lesions were visualized by β-galactosidase reaction products (blue). Note that the transplanted human cells are markedly labeled *in vivo* at the light microscopic level. (F) Antigenic phenotype of remyelinating cells in the lesions are P0 positive (peroxidase reaction, brown), and their nuclei are counterstained with hematoxylin (blue). P0 immunostaining demonstrates many Schwann-cell-like remyelination throughout the lesion (bar, A–C, 25 μm; D, 10 μm; E, 1 μm; F, 7 μm).

mm apart from control (Fig. 6B1), demyelinated (Fig. 6B2), and transplanted (Fig. 6B3) dorsal columns. The latencies of the responses in the demyelinated dorsal column (Fig. 6B2) are substantially delayed compared to controls (Fig. 6B1). However, following human neural precursor cell transplantation (Fig. 6B3) which led to extensive remyelination, the latencies are similar to controls. Conduction velocities were calculated for the three groups and are shown in Fig. 6C, indicating the restoration of conduction velocity in the transplanted group.

DISCUSSION

In this study we demonstrate that clonally expanded multipotential neural progenitor cells from the adult human brain can form functional Schwann cell-like myelin when transplanted into the demyelinated rat spinal cord. These progenitor cells expressed nestin and were self-renewing in culture until induced to differentiate by removing mitogens from the culture. Antigenic analysis after mitogen removal in culture revealed the differentiation into both neuron- and glia-like cells. In general, neurons, astrocytes, and a low number of oligodendrocytes and Schwann cells were present in the mitogen-free cultures. Following transplantation into the demyelinated rat spinal cord, however, the vast majority of cells differentiated into a peripheral-type of myelin-forming cell which produced functional myelin.

Clonal Expansion of Multipotential Adult Human Neural Precursor Cells

Both proliferation and differentiation of the clonal multipotential neural precursors derived from the

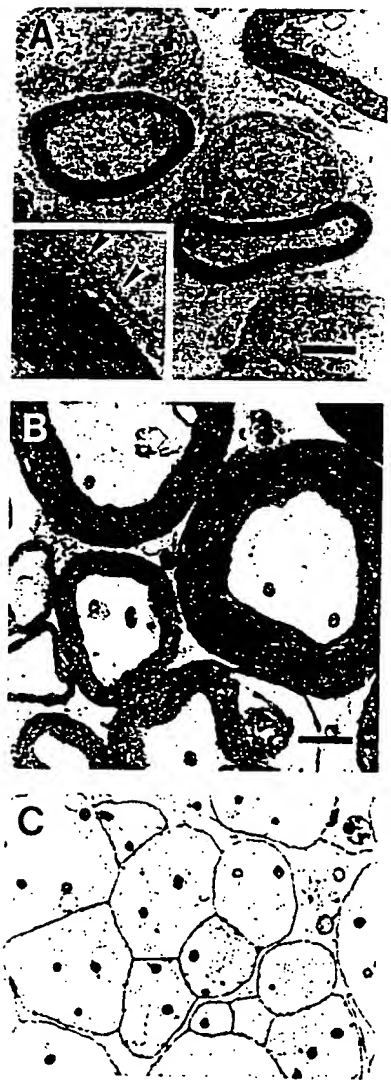

FIG. 5. All demyelinated spinal cords that received adult human precursor cell injections showed clear evidence of remyelination (A) of the demyelinated axons in electron micrographs. Examination at higher magnification showed the presence of a basal lamina surrounding the fibers (arrowheads). The large cytoplasmic and nuclear regions of the cell and the presence of a basal lamina indicate a peripheral pattern of myelination. Normal (B) and demyelinated (C) axons in the dorsal columns at the electron microscopic level. Bar, 1 μm.

adult brain could be controlled relatively efficiently. Several lines of evidence indicate that the cells in the clones are composed of a common multipotential cell rather than separate committed precursors. First, the proportion of neurons generated is independent of passage number, suggesting that the cellular properties are constant as the clones expand. This stability is supported by the unchanged differentiation capacity in clones of acutely dissociated cells and in subclones. Second, subcloning experiments demonstrate that multipotential secondary clones can be derived from a single primary clone, again showing the multipotentiality of single clonal cells. Asymmetric cell division may still be an important mechanism for cell-type specification *in vivo* (11). However, a strict asymmetric model, in which only one of the daughter cells maintains multipotentiality, cannot account for the exponential increase in the neural precursor cells seen in our cultures.

Differentiation of Neural Precursor Cells into Morphologically Defined Schwann-like Cells

In the normal CNS axons with oligodendrocyte-associated myelination do not have large nuclear and cytoplasmic surrounds, nor do they have an associated basement membrane. Rather, the cell bodies of the oligodendrocyte are relatively small and remote from the site of axonal myelination. However, following transplantation of the clonal neural precursor cells into a demyelinated and aglial region of the spinal cord *in vivo,* extensive differentiation into myelin-forming cells with morphological and phenotypical characteristics of Schwann cells was observed. These cells exhibited the hallmark characteristics of peripheral myelin-forming cells, large nuclear and cytoplasmic regions surrounding the axons which in turn were covered by a basement membrane (3), and immunohistochemical analysis demonstrated that most of them were P0 positive. Keirstead *et al.* (28) recently demonstrated that neural precursor cells derived from the neonatal rat brain and immunoselected for glial commitment can produce P0-positive myelin-forming cells *in vivo.* We cannot rule out the possibility that some neuronal or glial differentiation occurred, because we observed a few NSE-positive or GFAP-positive cells in the EB-X lesion. Moreover, some of the myelinated profiles were more characteristic of oligodendrocyte myelination. Future quantitative immunohistochemical studies on these tissues will be important to more fully characterize the phenotypes of the myelin-forming cells after transplantation. However, the abundance of cells with distinct morphological and immunohistochemical features characteristic of peripheral myelin-forming cells (large nuclear and cytoplasmic regions around the axons surrounded by a basement membrane and their P0 positivity) clearly indicate that the transplanted precursor cells differentiated into a peripheral pattern of myelin-forming cells.

It is well established that peripherally derived Schwann cells can myelinate the spinal cord which is normally myelinated by oligodendrocytes (4, 14). Given that endogenous remyelination by Schwann cells can occur in the spinal cord in certain circumstances, it was important to ascertain that the myelin-forming cells were derived from the donor source and not from endogenous invasion of Schwann cells from the periphery. To address this issue we used a model system that prevents endogenous invasion of peripheral Schwann cells for 6 to 8 weeks; we studied the cells about 3

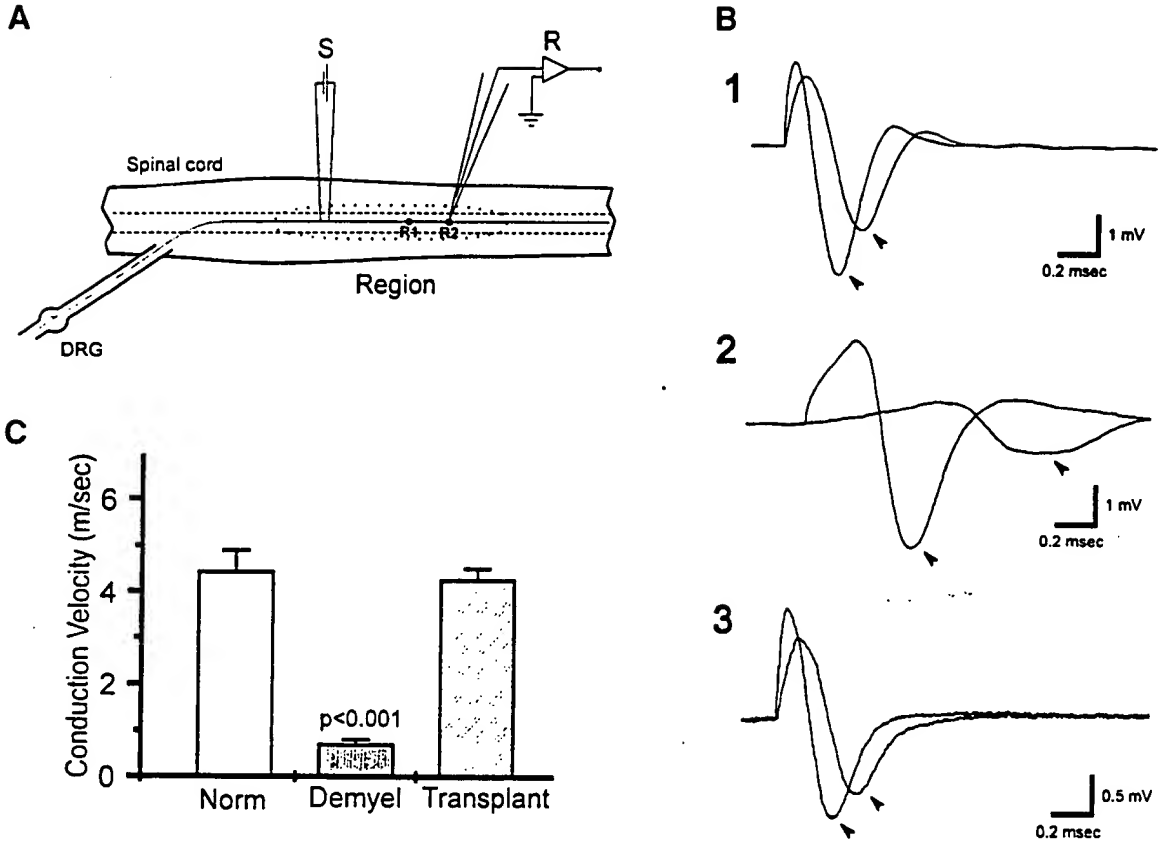

FIG. 6. (A) Schematic showing the dorsal surface of spinal cord with the positions of the stimulating (S) and recording (R) electrodes. Shaded region indicates the area of demyelination or remyelination. (B) Compound action potentials recorded at 1-mm increments along the dorsal columns in control (1), EB-X demyelinated (2), and transplant-induced remyelinated (3) axons. (C) Conduction velocity for the three groups (n = 5, each group) of axons recorded at 26°C. Bars, SEM.

weeks after transplantation which is well within the time window where no endogenous myelination occurs (4). The model utilizes X-irradiation to block host cell division followed by injections of ethidium bromide to chelate nucleic acids and kill the glial cells within the lesion zone. Moreover, in a limited number of experiments we transplanted LacZ-transfected donor progenitor cells and found X-Gal reactivity in cells exhibiting a peripheral myelination pattern. The extensive differentiation of neural precursor cells into Schwann cell-like cells in our studies is not likely the result of Schwann cell contamination in our cultures. The cells were derived from single cell clones showing homogenous properties and the capacity to differentiate into either neurons or glia.

Kalyani *et al.* (27) suggest that appropriate manipulation of culture conditions (45, 51) could promote embryonic neuroepithelial cell differentiation into more restricted CNS and PNS neural precursor cells. Indeed, embryonic neuroepithelial cells derived from the spinal cord have been reported to give rise to PNS elements and to other cell types in the body including skin melanocytes (24, 31, 41). More recently, Mujtaba *et al.* (40) provide evidence for a common neural progenitor cell derived from the embryonic spinal cord for the CNS and the PNS. Our results suggest that such a multipotential precursor may also be present in the adult human brain and that transplantation of these cells into the appropriate pathological environment of the adult CNS can allow Schwann cell differentiation and functional remyelination *in vivo*.

Why Do Neural Precursor Cells Derived from Adult Brain Differentiate into Schwann-like Cells in CNS in Vivo?

The Schwann cell differentiation *in vivo* may be the result of both the intrinsic capacity of the progenitor cells and the cellular and extracellular milieu of the transplant zone. In the EB-X lesion it is important to note that not only does this model lead to long-lasting demyelination, but all cellular elements inclusive of astrocytes and oligodendrocytes are killed within the lesion thus rendering the white matter tract agliotic and enriched in axons. The dominant cellular elements in the lesion are naked axons and macrophage-like scavenger cells in the glial-free environment. A large body of work indicates that axon-associated signals

may be important in aspects of Schwann cell differentiation (12, 43, 55). The development and maintenance of cell types are dependent upon and influenced by a number of intrinsic genetic factors as well as environmental signals (6, 47, 49, 54). Such signals may be provided by cell-to-cell contact, electrical stimulation, or the secretion of neurotransmitters and neurotrophic factors (9).

It should also be pointed out that interleukins released from macrophages have been implicated in Schwann cell differentiation following peripheral nerve injury (21, 32). It is conceivable that cytokines released from microglia or macrophages in the lesion site could contribute to the massive Schwann cell differentiation following neural precursor cell transplantation. It is possible then that the neural precursor cells derived from the adult human brain and transplanted into an axon-enriched environment *in vivo* in the absence of potential trophic influences from surrounding glia and neurons biases the differentiation of the neural precursor cells to a more restricted PNS lineage. It is important to note that EGF-responsive neural stem cells derived from fetal rodents formed an oligodendrocyte pattern of remyelination in myelin-deficient rats (20). This may result from differences in fetal and adult sources of the cells or a species difference. Another possibility is that the myelin-deficient rat, which has an abundance of astrocytes around the amyelinated axons, could provide a trophic influence for the differentiation of oligodendrocytes. It is not clear what the differences are between known totipotential stem cells derived from embryos and less defined progenitor cells derived from adult subependymal zone. It will be interesting to determine if embryonically derived stem cells form peripheral or central patterned myelin when transplanted into the adult demyelinated CNS.

Potential of Neural Precursor Cells to Repair the Damaged CNS

Neurons are not generated in large numbers in the adult mammalian CNS with the exception of the olfactory bulb (1, 34, 36) and the hippocampal formation (2, 7). Moreover, Gould *et al.* (18) have made the intriguing observation that learning can enhance neurogenesis in the adult hippocampus, possibly by differentiation of precursor cells. Cultured precursors derived from adult mice have been shown to differentiate into neurons and glia, but little is known about the mechanisms that regulate the differentiation of these cells (19). Both the clonal analysis and the response to growth factors reported here show that neural precursor cells derived from the adult human CNS have some properties that are similar to stem cells derived from embryonic neuroepithelium. Further delineation of similarities and differences between embryonic and adult precursor cells is certainly important. While both share some similarities it is not clear that adult-derived cells are totipotent as are true stem cells derived from embryos, thus indicating the importance of investigations of both embryonic and adult brain-derived precursor cells.

While oligodendrocytes are the cells that normally myelinate CNS axons, peripheral myelin-forming cells such as Schwann cells (4, 22) and olfactory ensheathing cells (15, 23) can myelinate CNS axons *in vivo* and restore near normal conduction properties (22, 23). Peripheral myelin-forming cells may have the advantage if used as a cell therapy in multiple sclerosis (MS) patients of not having the antigenic properties of oligodendrocytes which elicit an immune response in MS patients (22). Harvesting sufficient numbers of Schwann cells from peripheral nerve biopsy and cell expansion is problematic. However, the development of human clonal neural precursor cells derived from either embryonic or adult CNS may allow for an abundant source of myelin-forming cells. Zhang *et al.* (56) have demonstrated that fetal neural stem cells can be treated to establish self-renewing pre-O2-A progenitors which form extensive oligodendrocyte myelination when transplanted into the myelin-deficient neonatal rat. Recently, Brustle *et al.* (6) have demonstrated that human embryonic stem-cell-derived glial precursors can be used as a source of myelinating transplants. Advances in the cell biology of progenitor cells derived from embryonic, fetal, or adult CNS open the prospect of developing cell lines as a potential source of a cell therapy for demyelinating diseases.

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Transplantation of adult rat hippocampus-derived neural stem cells into retina injured by transient ischemia

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Abstract

Neural stem cells are capable of differentiating along multiple central nervous system cell-type lineages, and their use as graft material has provided new strategies for the treatment of neuronal damage. We transplanted adult rat hippocampus-derived neural stem cells into eyes of adult rats that underwent ischemia-reperfusion injury. As control, the cells were also injected into normal rats eyes without ischemic insult. The rats were sacrificed at 1, 2, 4, and 8 weeks, and the eyes were examined histochemically. In eyes with the insult, the transplanted cells were well integrated into the host retinas and expressed Map2ab. In the control, none of the cells migrated into the retina. These results suggest that neural stem cells may be used as donor cells for transplantation to repair ischemic-injured retina.

Keywords: Retina; Neural stem cell; Ischemia-reperfusion; Transplantation; Rat; Animal model

Traditionally, retinal impairments by neuronal death or axonal severance have been considered incurable in humans and adult experimental mammals because the central nervous system (CNS) of adult mammals does not have a regenerative capacity. Although a number of attempts to repair damaged retinas using grafts of retinal tissue have been reported [3,5,6,11,14], they have encountered serious problems such as limited incorporation of grafted cells into the host retina and difficulty in supplying enough donor cells as has been already discussed [2,4,7,9,17,18,20]. Thus, the transplantation of retinal tissue is not promising as a therapeutic strategy for the treatment of retinal impairments from neuronal death at present.

The recent advances in the field of neural stem cells have brought great expectation that severe CNS damages can be repaired by using stem/progenitor cells [4,7,13,17]. It has been shown that transplanted neural progenitor cells, even heterotypical, can integrate with the host brain tissue and differentiate into appropriate neurons [15]. For the retina, an earlier study demonstrated that transplanted adult rat hippocampus-derived neural stem cells (AHSCs) can be integrated into the host retina in normal neonatal rats [18]. Later studies showed that AHSCs were integrated into the host retina even in mature rats in genetically-degenerated retinas [20] and in mechanically-injured retinas [9]. These results have encouraged the development of novel therapies for treating retinal impairments using neural stem cells.

The in vivo retinal ischemia-reperfusion model is a standard experimental model that has been used to investigate the damage of the retina induced by transient ischemia. By inducing transient ischemia with high intraocular pressure, this model can avoid direct mechanical injury to the retina and optic nerve, and provide high reproducibility [1,12,19]. We transplanted AHSCs into eyes that had been damaged by ischemia-reperfusion. This report is the first study to perform neural stem cell transplantation into eyes with acquired retinal disease besides mechanical injury.

The preparation of AHSCs has been described in detail [10,18]. In brief, hippocampal progenitor cultures were prepared from the hippocampus of adult Fisher rats. The dissociated cells were cultured on polyornithine/laminin-coated dishes using a mixture of Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1:1) supplemented with N2 (Gibco) and 20 ng/ml of recombinant human basic

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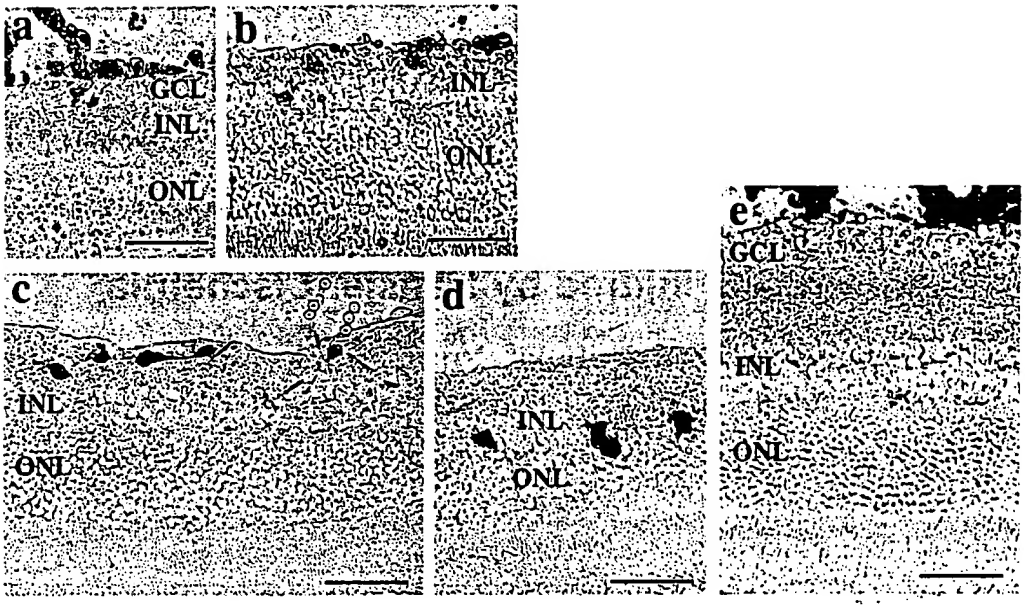

Fig. 1. Light microscopic photographs of host retinas demonstrating the transplanted AHSCs stained dark blue for β-Gal. In retinas with ischemia-reperfusion insult (a–d), prominent cell losses occurred primarily in the ganglion cell layers and inner nuclear layers as compared with the retina without the insult (e). (a) At 1 week after transplantation, the intravitreally injected AHSCs can be seen in the ganglion cell layer. (b) At 2 weeks after transplantation, the grafted AHSCs are seen in the inner nuclear layer. (c, d) At 8 weeks, the transplanted cells can be seen in various layers of the inner retina, and some of them seem to be process-bearing cells (c). (e) A control retina 2 weeks after transplantation with no ischemic insult. Intravitreally injected AHSCs are not present in the host retina. INL, inner nuclear layer. ONL, outer nuclear layer. Scale bars represent 50 μm.

fibroblast growth factor (bFGF) (Genzyme). Isolated stem cells were genetically marked with β-galactosidase (β-Gal) and cloned. The PZ5 clone, previously characterized extensively [10], was used. The cultured and harvested cells were washed with DMEM/Ham's F12 and suspended at a density of 100,000 cells/μl in DMEM/Ham's F12 for transplantation.

Adult (8–12 week old) Fisher rats were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg), and the pupils were dilated with topical 0.5% phenylephrine hydrochloride and 0.5% tropicamide in order to monitor the ocular fundi. Transient retinal ischemia was induced by raising the intraocular pressure to 110 mmHg for 60 min (see Ref. [12]). Immediately after beginning the reperfusion, the AHSCs were injected into the vitreous cavity of the treated eyes under trans-pupillary observation using a binocular surgical microscope. The injection was made with a 10-μl Hamilton microsyringe with a 30-gauge beveled needle. A total of 500 000 cells in 5 μl of DMEM/Ham's F12 were injected. For control, the cells were also injected into eyes with no ischemic insult.

The rats were sacrificed 1, 2, 4, and 8 weeks after the transplantation ($n = 4$ for the ischemic group and $n = 3$ for the control group at each time point), and the eyes were processed for histochemical studies.

In situ staining of grafted cells for β-Gal: all of the enucleated eyes except the two described below were fixed in 2% paraformaldehyde, 0.1% glutaraldehyde, 0.02% NP-40, and 0.01% deoxycholate in PBS. After 1 h, the anterior segments were removed, and the β-Gal staining was done by placing the eye cups in a solution of 2.5 mM X-Gal, 5 mM potassium ferricyanate, 5 mM potassium ferro-

Fig. 2. Confocal images of the retina 4 weeks after AHSCs transplantation with ischemic insult. (a) Anti-Map2ab immunoreactivity. (b) Anti-β-Gal immunoreactivity. (c) The merged image of anti-Map2ab and anti-β-Gal immunoreactivity, showing the grafted AHSCs differentiate into mature neurons. INL, inner nuclear layer. ONL, outer nuclear layer. Scale bar represent 50 μm.

cyanate, 2 mM $MgCl_2$ in PBS [16] at room temperature overnight. The stained tissue was washed with PBS and soaked in PBS containing 25% sucrose for cryoprotection. Ten-micrometer sections were cut on a cryostat and examined with a light microscope.

Immunostaining: two eyes of two animals sacrificed 4 weeks after ischemic insult were fixed in 4% paraformaldehyde, cryoprotected, and sectioned at 10 μm on a cryostat. Double immunostaining was performed on these sections. The sections were processed for anti-β-Gal (1:500, Promega) and anti-Map2ab (1:500, Sigma), and followed by reaction with FITC-conjugated secondary antibody (1:100, DAKO) to anti-β-Gal and rhodamine-conjugated antibody (1:100, DAKO) to anti-Map2ab. Confocal microscopy was used to study these sections.

In every treated eye, transplanted cells were readily identified by the transgenic β-Gal marker. In the eyes that underwent ischemia-reperfusion insult, prominent cell loss were observed primarily in the ganglion cell layer and inner nuclear layer (Fig. 1). The changes were typical of the findings in ischemia-reperfusion insult described earlier [12].

In eyes with the ischemia insult, the intravitreally-injected AHSCs invaded the retinal ganglion cell layer by 1 week after the transplantation (Fig. 1a) and were identified in the retinal inner nuclear layer 2 weeks after the transplantation (Fig. 1b). At 4 weeks, the donor cells were integrated into the host retina and expressed Map2ab (Fig. 2) which indicated that the AHSCs had differentiated into mature neurons. At 8 weeks after the transplantation, the transplanted cells were integrated into the different layers of the inner retina (Fig. 1c,d) and appeared as process-bearing cells (Fig. 1c). A number of transplanted cells were detected within the vitreous cavity but the number decreased with increasing postinjection times (Fig. 1).

In the control animals without ischemic insult, AHSCs did not invade the host retina (Fig. 1e). Although many of the transplanted cells were observed to survive in the vitreous cavity at 2 weeks after transplantation, the numbers decreased at 4 weeks and were mostly gone at 8 weeks after the transplantation (data not shown).

These results demonstrated that intravitreally-injected AHSCs migrated and integrated into the host retinas of adult rats that had undergone ischemic insult while none of the cells migrated into the retina without ischemic insult. In the host retinas, many cells in the ganglion cell layer and inner nuclear layer were lost after the ischemic insult. The transplanted AHSCs migrated primarily into these layers and differentiated into mature neurons replacing some of the lost cells.

The observation that AHSCs did not enter the host retina in the control adult rats agrees with previous findings [18]. The question then arises as to why retinas damaged by ischemia will accept the migration and integration of transplanted neural stem cells while normal adult retinas will not. First, it is likely that some types of trophic factors or cytokines that promote survival, migration, and neuronal differentiation of the transplanted stem cells are produced in the retina that had been injured by ischemia. It has already been reported that bFGF, which is known to be important for survival of AHSCs [10], is up-regulated in retinas after transient ischemic insult [8]. In addition to bFGF, other factors were probably up-regulated in the ischemic retina to stimulate migration and neural differentiation of the grafted cells.

Secondly, it is highly likely that serous components enter the retina because the blood–retina barrier is broken by the ischemic insult [19]. Such serous components, as well as intrinsically expressed factors, can promote survival, migration, and neuronal differentiation of the transplanted AHSCs. In the control animals, it is likely that the absence of such factors prevented the integration of the grafted cells into the host retina, and was probably the cause for the decrease of surviving AHSCs in the vitreous after the transplantation.

Another factor that might promote the migration of the transplanted cells is a disruption of the retinal internal limiting membrane. The retinal internal limiting membrane can be a barrier to cell invasion under normal conditions, but it could be interrupted by the ischemia. In the present study, we suggest that disruptions of the retinal internal limiting membrane by ischemia allowed the AHSCs to enter the retina.

This experimental model of retinal injury by transient retinal ischemia induced by raising the intraocular pressure can be considered comparable to an acute glaucomatous attack, a central retinal artery occlusion, or an ischemic optic neuropathy. In these diseases, it has been generally believed that it is not possible to repopulate the lost retinal cells and repair the retinal injury. The present results have shown that intravitreally injected AHSCs can partly repopulate the lost host cells and differentiate into a neuronal lineage. However, it still is not known whether the transplanted cells can establish a functional network with host neural circuitry and acquire proper functions as retinal neurons. Nevertheless, our results suggest that neuronal stem cells are good candidates to reconstruct the neural circuitry of ischemic-injured retina, and show the potentiality of therapeutic transplantation using neuronal stem cell on retinal impairments that are generally regarded as incurable.

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Incorporation and Differentiation of Hippocampus-Derived Neural Stem Cells Transplanted in Injured Adult Rat Retina

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PURPOSE. In a previous study it has been shown that adult rat hippocampus-derived neural stem cells can be successfully transplanted into neonatal retinas, where they differentiate into neurons and glia, but they cannot be transplanted into adult retinas. In the current study, the effect of mechanical injury to the adult retina on the survival and differentiation of the grafted hippocampal stem cells was determined.

METHODS. Mechanical injury was induced in the adult rat retina by a hooked needle. A cell suspension (containing 90,000 neural stem cells) was slowly injected into the vitreous space. The specimens were processed for immunohistochemical studies at 1, 2, and 4 weeks after the transplantation.

RESULTS. In the best case, incorporation of grafted stem cells was seen in 50% of the injured retinas. Most of these cells located from the ganglion cell layer through the inner nuclear layer close to the injury site. Immunohistochemically, at 1 week, more than half of the grafted cells expressed nestin. At 4 weeks, some grafted cells showed immunoreactivity for microtubule-associated protein (MAP) 2ab, MAP5, and glial fibrillary acidic protein (GFAP), suggesting progress in differentiation into cells of neuronal and astroglial lineages. However, they showed no immunoreactivity for HPC-1, calbindin, and rhodopsin, which suggests that they did not differentiate into mature retinal neurons. Immunoelectron microscopy revealed the formation of synapse-like structures between graft and host cells.

CONCLUSIONS. By the manipulation of mechanical injury, the incorporation and subsequent differentiation of the grafted stem cells into neuronal and glial lineage, including the formation of synapse-like structures, can be achieved, even in the adult rat retina. (*Invest Ophthalmol Vis Sci.* 2000;41:4268-4274)

Since the mid-1990s, it has been possible to isolate neural stem or progenitor cells from various parts of the central nervous system (CNS), such as the hippocampus, subventricular zone, spinal cord, and ependyma.[1-4] In general, these cells can expand in serum-free medium and proliferate in response to growth factors such as epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF). From a clinical point of view, they have some potential advantages for retinal transplantation compared with embryonic or newborn retinal cells. First, they can be expanded through numerous passages in vitro and frozen for storage. Second, they can be easily manipulated, such as by pretreatment with growth factors or gene transduction, before they are transplanted.

Adult rat hippocampus-derived neural stem cells, first isolated by Palmer et al.[5], are one of the few cell lines that have been shown by clonal analysis to have multipotency and self-renewability. In a previous study of ours, we found that the hippocampal stem cells could be successfully transplanted and integrated into the neonatal rat retina but that when they were transplanted into adult eyes, they aggregated on the surface but never migrated into the retina.[6]

In this study, for the purpose of assessing the possibility and limitations of the use of brain-derived neural stem cells for retinal transplantation, we investigated whether these hippocampal stem cells could migrate and become incorporated into mechanically injured adult rat retinas.

MATERIALS AND METHODS

Preparation of Cells for Grafting

LacZ-labeled clonal adult rat hippocampus-derived neural stem cells (clone PZ5, kindly provided by Fred H. Gage, Salk Institute, La Jolla, CA) were used in this study. They were cultured on laminin/poly-L-ornithine-coated dishes containing Dulbec-

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co's modified Eagles medium-Ham's F12 (DMEM-F12; Gibco, Rockville, MD) supplemented with N2 (Gibco) and 20 ng/ml bFGF (Genzyme, Cambridge, MA), and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After having been subcultured for 2 weeks to 3 months, they were harvested for grafting with 0.05% trypsin in DMEM-F12, washed with 0.01% trypsin inhibitor (Wako, Osaka, Japan) in DMEM-F12, and suspended at a density of 30,000 cells/μl in high-glucose Dulbecco's phosphate-buffered saline (D-PBS, Gibco) containing 20 ng/ml bFGF.

Animal Preparation and Grafting Procedure

Eight-week-old male Fischer rats (n = 30) were obtained from Shimizu Laboratory Supplies (Kyoto, Japan). All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were anesthetized with a mixture (1:1) of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg) administered intramuscularly. The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine eye drops. The corneas were anesthetized with drops of 0.4% oxybuprocaine hydrochloride. The eyeballs were perforated at the equator with a 27-gauge needle. A hooked 30-gauge needle was then inserted through the wound, and the retina was injured by scratching it parallel to the equator between the retinal vessels under direct observation with a surgical microscope equipped with a plano-concave contact lens for rats (Kyocon, Kyoto, Japan). Special care was taken to injure the whole layer of the retina, and success was affirmed by a small amount of subretinal bleeding. After the injury, 3 μl of the cell suspension (containing 90,000 cells) was slowly injected into the intravitreal space with a microsyringe fitted with a 30-gauge blunt needle (15 rats, 30 eyes). As a control, 3 μl of the cell suspension was injected into the intravitreal space of noninjured eyes (15 rats, 30 eyes). The results from five eyes of the control group were excluded due to complications of massive vitreous hemorrhage.

Tissue Sectioning

The animals were anesthetized by inhalation of diethyl ether and fixed by transcardial perfusion with 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (PB) 1, 2, and 4 weeks later. The eyes were enucleated to make eyecups. The eyecups were immersed in the same fixative for 2 hours at 4°C and then in 15%, 20%, and 25% sucrose-PBS for cryoprotection. They were embedded in optimal cutting temperature compound (OCT; Miles, Elkhart, IN) after adjustment of their horizontal planes parallel to the cutting plane, and 20-μm frozen sections were made in a cryostat. Continuous sections including the injury site were cut for each eye.

Immunocytochemistry

The specimens were washed with 0.1 M PB and then incubated with 20% skim milk (Dainihon-Seiyaku, Osaka, Japan) in 0.1 M PB containing 0.005% saponin (0.1 M PB-saponin; Merck) for 10 minutes to block nonspecific antibody binding. They were then incubated with primary antibodies diluted in 5% skim milk in 0.1 M PB-saponin for 24 hours at 4°C. Antibodies and concentrations used in this study were as follows: mouse monoclonal anti-β-galactosidase (β-gal, 1:1000; Promega, Madison, WI), rabbit polyclonal anti-β-gal (1:5000; Chemicon, Temecula, CA), mouse monoclonal anti-nestin (1:1000; PharMingen, San Diego, CA), mouse monoclonal anti-microtubule associated protein (MAP) 2ab (1:100; Sigma, St. Louis, MO), mouse monoclonal anti-MAP5 (1:1000; Chemicon), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:1000; Chemicon), rabbit anti-myelin basic protein (MBP; 1:500; UltraClone, Wellow, UK), mouse monoclonal anti-HPC-1 (1:1000; Sigma), mouse monoclonal anti-calbindin (1:500; Sigma), and rabbit anti-rhodopsin (1:1000; LSL, Tokyo, Japan).

After the reaction with primary antibodies, the specimens were washed with 0.1 M PB-saponin and incubated with secondary antibodies diluted in 5% skim milk in 0.1 M PB-saponin for 90 minutes. Antibodies and concentrations used in this study were as follows: fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulin (1:100; Amersham, Buckinghamshire, UK), FITC-conjugated donkey anti-rabbit immunoglobulin (1:100; Amersham), Cy5-conjugated goat anti-mouse IgG (1:200; Amersham), and Cy5-conjugated donkey anti-rabbit IgG (1:200; Amersham).

Sections were then washed with 0.1 M PB, mounted with glycerol-PBS (1:1) and observed with a laser-scanning confocal microscope (1024; Bio-Rad, Hercules, CA).

Immunoelectron Microscopy

Immunoelectron microscopy using the silver-enhancement technique was done as described.[7] Briefly, after having been blocked with 20% skim milk in 0.1 M PB-saponin, the sections were incubated with the anti-β-gal antibody (1:1000; Promega) and subsequently with an anti-mouse IgG antibody coupled with 1.4-nm gold particles (1:50; Nanoprobes, Stony Brook, NY). After the sections had been washed, they were fixed with 1% glutaraldehyde (Nacalai Tesque, Kyoto, Japan) in 0.1 M PB for 10 minutes, and the sample-bound gold particles were then silver-enhanced at 20°C for 12 minutes by use of an HQ-silver kit (Nanoprobes). They were again washed and postfixed with 0.5% osmium oxide (Nacalai Tesque) in 0.1 M PB at pH 7.3, dehydrated by passage through a graded series of ethanol (50%, 60%, 70%, 80%, 90%, 95%, and 100%), and embedded in epoxy resin. From these samples, ultrathin sections were cut, stained with uranyl acetate and lead citrate, and then observed with an electron microscope (JEM-1200EX; JEOL, Tokyo, Japan).

Results

Incorporation and Distribution of Grafted Cells

In an attempt to elucidate the efficacy of transplantation of hippocampal stem cells into the adult rat retina, we injected them into the vitreous space. The stem cells were labeled with the *LacZ* gene retrovirally, so that we could identify β-gal-immunoreactive cells as the grafted cells. In our previous study, we confirmed that β-gal enzyme leaking from damaged or dead grafted cells was not taken up by host retinal cells.[6]

First, we compared the incidence of eyes with incorporated grafted cells between the injured group and the noninjured group. In the injured group, 1 week after transplantation, β-gal-immunoreactive cells were incorporated into the host retina in 10% of the experimental eyes (1 of 10). At 2 and 4 weeks, the percentage of eyes with incorporated cells increased to 50% (5 of 10) and 40% (4 of 10), respectively (Table 1). In the eyes with incorporated grafted cells, the grafted cells

TABLE 1. Incidence of Eyes with Incorporated Grafted Cells

	1 Week	2 Weeks	4 Weeks
Injured group	1/10	5/10*	4/10*
Noninjured group	0/5	0/10	0/10

Values are number of eyes with incorporated grafted cells/total surgically treated eyes. Five rats were used in each experiment. The results from five eyes of the noninjured group at 1 week were excluded because of complications of massive vitreous hemorrhage.

* Incidence in the injured group was significantly higher than that in the noninjured group (Fisher's exact probability test, $P < 0.05$).

were distributed around the site of injury, where GFAP immunoreactivity of the host retina was upregulated (Fig. 1A). In contrast, no eyes incorporated grafted cells in the noninjured group at any period after transplantation (Table 1). The grafted cells were found to have aggregated on the inner surface but never to have been incorporated into the host retina of the noninjured group (Fig. 1B). Statistical analysis by Fisher's exact probability test showed a significant difference ($P < 0.05$) between the injured and noninjured groups in the incidence of successful incorporation of the grafted cells at both 2 and 4 weeks after transplantation.

The pattern of grafted cell distribution was almost the same at all times after the injection. The grafted cells were observed not only at the site of injury where normal retinal structure was destroyed, but also in the surrounding area where the normal retinal structure was retained. Most of them were situated in the inner nuclear layer (INL) with some in the ganglion cell layer (GCL), where they formed a layer-like structure. A few grafted cells were found on the inner surface of the retina and in the outer nuclear layer (ONL). The width of distribution of the incorporated grafted cells ranged between 790 μm and 1200 μm around the site of injury (data not shown). This width was much greater than that of the actual injury in all cases, which was less than 100 μm.

The grafted cells adherent to the inner surface of the host retina in the injured group were round and had no processes, whereas most incorporated cells had elongated processes, and some of them showed morphologies reminiscent of amacrine and bipolar cells (Fig. 2).

Immunohistochemistry on Sections after Transplantation

Immunohistochemical studies were performed on sections with incorporated grafted cells in the injured group. The sections were double immunostained with anti-β-gal antibody and antibodies against specific cell-type markers. The cell-type markers used were nestin for immature or undifferentiated cells, MAPs for neuronal lineage cells, GFAP for astrocytes and Müller cells, MBP for oligodendrocytes, HPC-1 for amacrine cells, calbindin for horizontal and some amacrine cells, and rhodopsin for rod photoreceptor cells. The ratios of double-stained cells to β-gal-positive cells were calculated to estimate the characteristics of the grafted cells after transplantation.

Our preliminary studies showed the presence of nestin immunoreactivity in more than 96% of the cultured hippocampal stem cells; however, no immunoreactivity for other specific markers of differentiated cell types, including MAP2ab, MAP5, GFAP, MBP, HPC-1, calbindin, and rhodopsin, was detected (data not shown).

Among the grafted cells, nestin-positive cells were over 50% at the end of 1 and 2 weeks after transplantation; however, they decreased to 36% after 4 weeks (Table 2, Figs. 3A, 3B, 3C). MAP5-positive cells increased markedly from 1% to 22% between 1 and 2 weeks, whereas MAP2ab-positive cells gradually increased from 1 to 4 weeks (Table 2, Figs. 3D, 3E, 3F). As for the two glial markers, GFAP-positive grafted cells increased from 2% to 10% between 2 and 4 weeks, but MBP-positive cells were hardly observed from weeks 1 through 4 (Table 2, Figs. 3G, 3H, 3I). Immunoreactivity for retinal cell markers, HPC-1, calbindin, and rhodopsin was hardly detected in the grafted cells throughout the 4 weeks (Table 2, Figs. 3J, 3K, 3L).

The immunoreactivity for nestin and GFAP was also observed in the host Müller cells around the sites of injury, where the grafted cells were incorporated into the host retinas (Figs. 3A, 3B, 3C, 3G, 3H, 3I).

Immunoelectron Microscopy on Sections at 4 Weeks after Transplantation

Immunoelectron microscopy was performed on sections of 4-week specimens. Grafted cells were identified by the presence of gold particles indicating immunoreactivity for β-gal. In

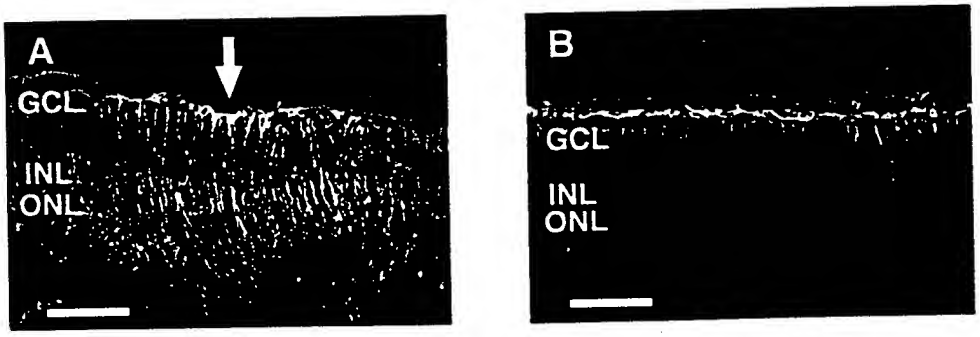

FIGURE 1. Double-label immunofluorescence study using antibodies against β-gal (*green*) and GFAP (*red*) in the injured group (A) and noninjured group (B) 2 weeks after the injection. (A) β-Gal-positive grafted cells were observed primarily in the GCL and INL in the host retina around the site of injury (*arrow*). Expression of GFAP in the host retina was upregulated. (B) β-Gal-positive cells were located on the inner surface of but not within the host retina. The expression of GFAP was localized in the astrocytes and the end feet of the Müller cells. Scale bar, 100 μm.

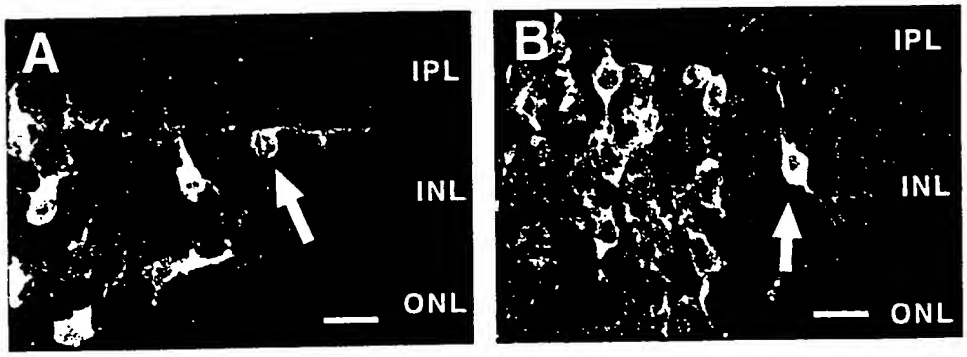

FIGURE 2. β-Gal-immunoreactive grafted cells, which are similar to amacrine (A, *arrow*) and bipolar (B, *arrow*) cells, 1 and 4 weeks after transplantation, respectively. Scale bar, 20 μm.

general, the grafted cells had heterochromatic nuclei and a large number of mitochondria (Figs. 4A, 4B).

In the inner plexiform layer (IPL) and at the innermost part of the INL, grafted cells were often found in a group (Fig. 4A). Some of them were irregular in shape and had pseudopodia that made contact with other grafted cells (Fig. 4A), which is a characteristic of actively migrating cells. Some other grafted cells had a relatively round shape and extended their processes to make close contact with host cells at the innermost part of the INL (Fig. 4B). At a higher magnification, symmetrical and asymmetrical membrane thickening, which represent puncta adherentia and synaptic junctions, respectively, were observed between graft and host cells (Figs. 4C, 4D) indicating that they formed close contacts with each other.

DISCUSSION

Neural stem cells are expected to be useful clinically for replacing damaged neurons or for ex vivo gene therapy.[8] In the field of brain science, they have been tested on damaged brain models[9,10] as cell resources for replacement therapy. Also in the field of ophthalmology, it is reported that neural stem cells could be successfully transplanted into damaged retina.[11-13] Therefore, it is important to assess the application of neural stem cells for retinal transplantation therapy.

This study has shown the ability of hippocampus-derived neural stem cells to migrate and differentiate in the injured retina. However, the limitation of their differentiation into authentic retinal neurons was also recognized.

Pattern of Incorporated Grafted Cells in the Host Retina

The incidence of the eyes with incorporated grafted cells increased between 1 and 2 weeks but did not change between 2 and 4 weeks. Some time may be required for the cells that have migrated onto the retinal surface to create graft-host contacts and to migrate into the host retina. This behavior of the grafted cells is consistent with the results of our previous study.[6]

The grafted cells were located around the injured sites, where the expression of nestin and GFAP in the host Müller cells was upregulated. The width of the distribution of the grafted cells was much greater than that of the injury (less than 100 μm) at any time point evaluated. We therefore speculate that the grafted cells migrated into the host retina not only from the injured site but also from the vitreous surface around the injured site where the host Müller cells were activated by the injury. This speculation was supported by our other experiments that hippocampal stem cells can also incorporate into chemically damaged retinas (data not shown). It has been reported that upregulation of the expression of nestin and GFAP in astrocytes or Müller cells occurred in the CNS including the retina after various types of damage.[14-18] It also has been shown that activated Müller cells express a number of cytokines such as bFGF, ciliary neurotrophic factor (CNTF), and transforming growth factor (TGF)-α.[19-22] It seems reasonable that the Müller cells that were activated by the mechanical injury may have played an important role in the migration and/or differentiation of the surviving grafted cells.

For the purpose of assessing the effect of retinal injury, we chose the vitreous cavity instead of the subretinal space for the site of injection of the neural stem cells. Subretinal injection itself causes retinal detachment and much damage to the retina.

Differentiation and Integration of the Grafted Cells

The hippocampal stem cells used as the grafted cells were confirmed by immunocytochemistry to be immature cells. Before grafting, most of them expressed nestin. However, once they were grafted, the number of cells expressing nestin decreased. On the contrary, the cells expressing MAPs and GFAP increased with time, which suggests differentiation of the stem cells into cells of the neuronal and astroglial lineages. Among the MAPs, MAP2ab is thought to be a late marker of neuronal

TABLE 2. Differentiation Ratio of the Incorporated Grafted Cells in the Injured Group

	Nestin	MAP2ab	MAP5	GFAP	MBP	HPC-1	Calbindin	Rhodopsin
1 week	56.4	3.6	1.1	3.4	0.0	1.6	0.0	0.0
2 weeks	55.0 ± 3.2	5.1 ± 3.5	21.9 ± 7.6	2.3 ± 1.5	0.6 ± 0.5	1.0 ± 1.0	0.0 ± 0.0	0.2 ± 0.4
4 weeks	35.9 ± 19.0	9.7 ± 0.8	25.1 ± 10.7	9.9 ± 4.7	0.8 ± 1.3	0.3 ± 0.6	0.5 ± 0.9	0.0 ± 0.0

Values at 2 and 4 weeks are mean ± SD and are the ratio of grafted cells double-stained with anti-β-gal.

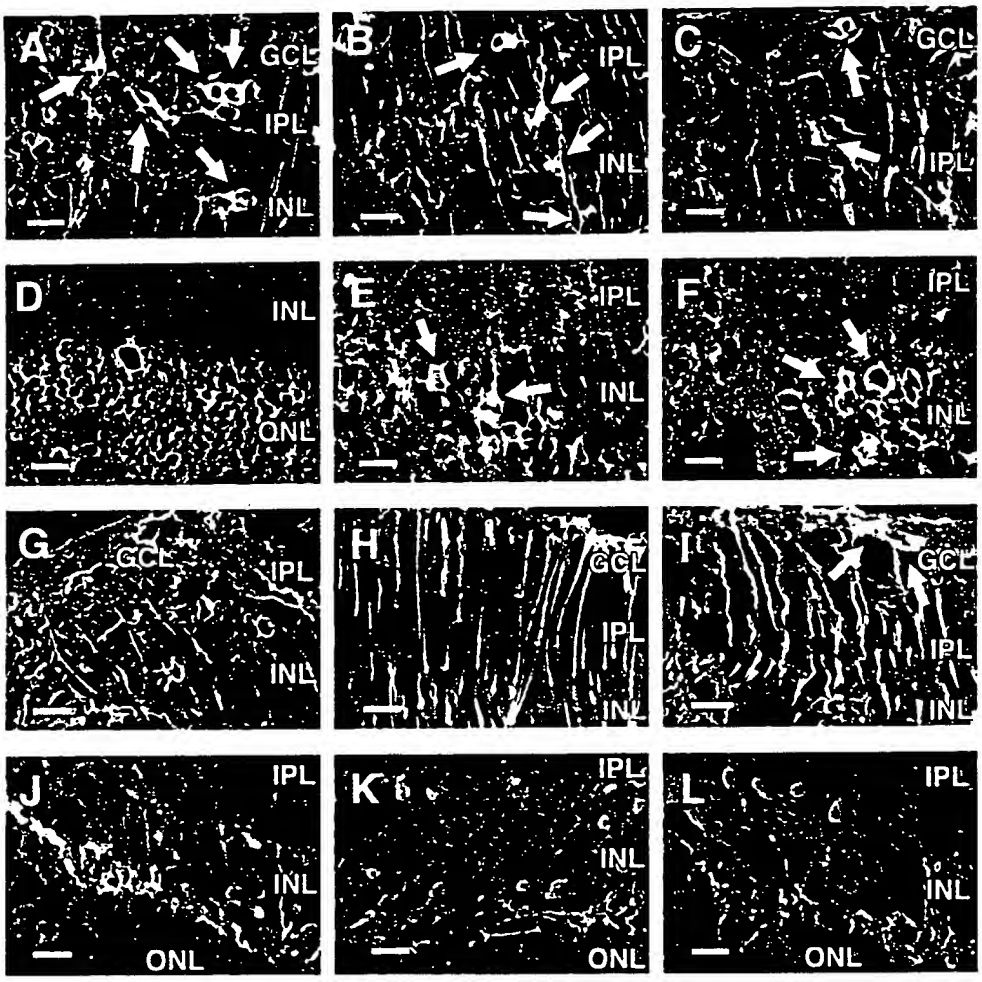

FIGURE 3. Double-label immunofluorescence at the end of 1 (A, D, G, and J), 2 (B, E, H, and K), and 4 (C, F, I, and L) weeks after cell transplantation. *Green*: β-Gal-immunoreactive cells; *red*: nestin- (A, B, and C), MAP5- (D, E, and F), GFAP- (G, H, and I), and calbindin- (J, K, and L) immunoreactive cells; *yellow*: double-stained cells (*arrows*). (A, B, and C) Nestin-positive grafted cells decreased in number from 1 to 4 weeks after transplantation. (D, E, and F) MAP5-positive grafted cells increased from 1 to 4 weeks after transplantation. (G, H, and I) Few GFAP-positive grafted cells are observed at 1 and 2 weeks after transplantation, but they begin to appear at 4 weeks. (J, K, and L) Calbindin-positive grafted cells are rarely observed at any time after the injection. Scale bar, 20 μm.

differentiation, because its expression increases as neuronal cells mature,[23] whereas the expression of MAP5 is generally abundant in neuronal cells at very early developmental stages.[23] These facts explain why the expression of MAP5 in the grafted cells increased earlier than that of MAP2ab. GFAP and MBP are markers for astrocytes and oligodendrocytes, respectively. The expression of MBP was hardly detected up to 4 weeks, whereas that of GFAP increased between 2 and 4 weeks after the grafting. This finding indicates that the hippocampal stem cells did not differentiate into oligodendrocytes but into astrocytes after the grafting, although they differentiated into both glial lineages in vitro.[5] It also suggests that the specific microenvironment in the retina, where no oligodendrocytes exist, may affect the fate of differentiation of the hippocampal stem cells. As for the retinal cell markers, HPC-1, calbindin, and rhodopsin, their expression in the grafted cells was hardly observed at any time after the grafting, indicating the failure of differentiation into retinal neurons even at the end of 4 weeks after the grafting. One possible reason for the failure is absence of unknown local cues in injured adult retina. There may be some unknown factors that are expressed only in earlier stages of retinal development and permit the hippocampal stem cells to differentiate into retinal neurons. Another possible explanation is limited plasticity of the hippocampus-derived neural stem cells. They may continue to possess the characteristics of cells in the hippocampus, from which they are derived, even after being transplanted into retinal tissue.

Immunoelectron microscopic study revealed the existence of graft-graft and graft-host contacts. The grafted cells formed puncta adhaerentia-like and asymmetrical synapse-like structures with the host cells. Not only mechanical contacts but also intercellular signaling could be formed between the graft and host cells. There are several reports describing graft-host synapse formation in the adult CNS in homotopic transplantation, such as retina to retina,[24,25] and also in heterotopic transplantation, such as retina to cerebellum.[26] It is still unknown whether these synapse-like structures actually function; however, the formation of such structures is significant evidence for integration of the grafted cells into the host retina.

Deriving Retinal Neurons from Neural Stem Cells

Further studies are needed to establish the utility of neural stem cells for replacement and reconstruction of retinal neurons. One possibility is retina-derived neural progenitor cells. A recent study revealed that embryonic retina-derived neural progenitor cells can differentiate into photoreceptors in vitro.[27] If they maintain the characteristics of retinal cells through expansion in vitro, they may differentiate into retina-specific neurons after transplantation. Another possibility is modification of cellular characteristics of the hippocampus-derived neural stem cells for retina-specific differentiation by transfection of key molecules such as homeobox genes.[28,29] Also, pretreatment of the neural stem cells with growth factors is a possible means of controlling the cells' fate. In fact, in our

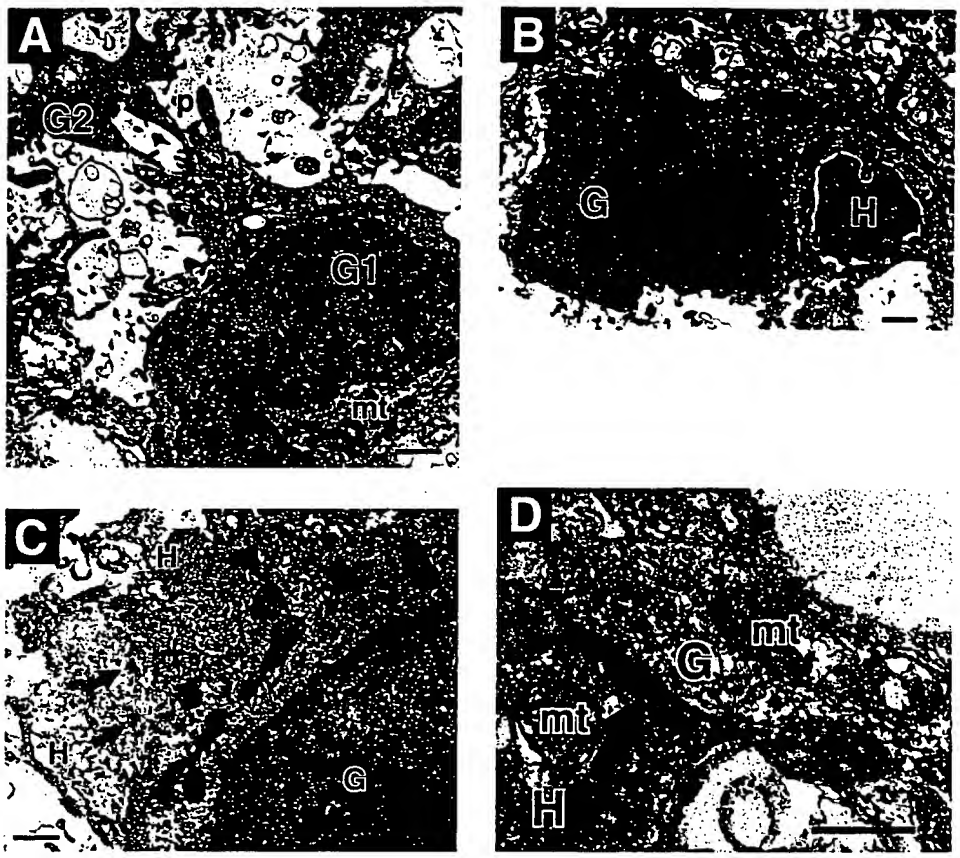

FIGURE 4. Immunoelectron microscopy on sections at 4 weeks after transplantation. (A) Grafted cells are identified by the presence of gold particles indicating immunoreactivity for β-gal. A gold-labeled grafted cell (G1) in the IPL extended its pseudopodia (p) and made contact with another grafted cell (G2). Note that the grafted cells contained a large number of mitochondria (mt). (B) A grafted cell (G) extended its process (*arrow*) and made close contact with a host cell (H) in the innermost part of the INL. (C) A grafted cell (G) in the INL formed contacts with host cells (H). Both symmetrical (*arrows*) and asymmetrical (*arrowhead*) membrane thickenings were observed. (D) An axon terminal of a grafted cell (G) labeled with gold particles (*small arrows*) in the IPL contained synaptic vesicles (*arrowheads*) and formed a synapse-like structure with a host cell (H). Postsynaptic density (*large arrow*) was observed in the host cell. Scale bar: 1 μm (A, B); 500 nm (C, D).

previous study, we found that some neurotrophins affect the differentiation of the hippocampal stem cells in vitro[30]; however, growth factors that can induce neural progenitor cells to produce retina-specific neurons have not yet been identified.

CONCLUSIONS

In conclusion, this study has yielded basic and important information regarding the transplantation of adult rat hippocampus-derived neural stem cells into the adult retina. First, incorporation of the grafted neural stem cells was achieved in injured adult retinas. Second, some of the incorporated neural stem cells showed differentiation into neuronal lineage and formed graft-host contacts such as puncta adhaerentia- and synapse-like structures. Third, even after successful transplantation and differentiation into cells of the neuronal lineage, the neural stem cells failed to differentiate into retina-specific phenotypes as shown by expression of HPC-1, calbindin, and rhodopsin, possibly because of their basic inability or an absence of local cues essential for differentiation into retinal neurons.

Acknowledgment

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Journal of Neuroscience Research 50:862–871 (1997)

Isolation and Transplantation of Multipotential Populations of Epidermal Growth Factor–Responsive, Neural Progenitor Cells From the Canine Brain

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Glial cell transplantation into myelin-deficient rodent models has resulted in myelination of axons and restoration of conduction velocity. The shaking (*sh*) pup canine myelin mutant is a useful model in which to test the ability to repair human myelin diseases, but as in humans, the canine donor supply for allografting is limited. A solution may be provided by self-renewing epidermal growth factor (EGF)-responsive multipotential neural progenitor cell populations ("neurospheres"). Nonadherent spherical clusters, similar in appearance to murine neurospheres, have been obtained from the brain of perinatal wildtype (*wt*) canine brain and expanded in vitro in the presence of EGF for at least 6 months. Most of the cells in these clusters express a nestin-related protein. Within 1–2 weeks after removal of EGF, cells from the clusters generate neurons, astrocytes, and both oligodendroglial progenitors and oligodendrocytes. Transplantation of lacZ-expressing *wt* neurospheres into the myelin-deficient (*md*) rat showed that a proportion of the cells differentiated into oligodendrocytes and produced myelin. In addition, cells from the neurosphere populations survived at least 6 weeks after grafting into a 14-day postnatal *sh* pup recipient and at least 2 weeks after grafting into an adult *sh* pup recipient. Thus, neurospheres provide a new source of allogeneic donor cells for transplantation studies in this mutant. J. Neurosci. Res. 50:862–871, 1997.

Key words: epidermal growth factor; myelin deficient; neural progenitor cells; glial cell transplantation

INTRODUCTION

Accumulating research in animal models raises hopes of using glial transplantation in therapy for human myelin diseases. Glia from a range of sources can myelinate axons in various recipient environments (reviewed in Duncan and Milward, 1995; Franklin and Blakemore, 1995; Duncan, 1996). Most research uses rodent recipients with inherited myelin disorders (reviewed in Duncan and Milward, 1995; Duncan, 1996) or with chemically induced demyelinating lesions (Blakemore and Crang, 1983; Blakemore et al., 1995), but there are obvious limitations to extending such studies to humans. Myelin diseases such as multiple sclerosis may develop over years, whereas most rodent myelin mutants die before adulthood. Glial cells grafted into rodents can migrate substantial distances (1–2 cm) but may need to travel much farther to reach surgically inaccessible lesions in humans. Mechanisms of differentiation, myelination, or remyelination may also differ between species. Most studies on human glia have not found close resemblances to rat oligodendroglial growth factor responses, antigenic profiles, or adult progenitor cells, although some similarities do exist (Kennedy et al., 1980; Dickson et al., 1985; Yong et al., 1988; Aloisi et al., 1992; Armstrong et al., 1992; Yong and Antel, 1993; Gogate et al., 1994; Satoh and Kim, 1994; Scolding et al., 1995).

The canine shaking (*sh*) pup model may help bridge the gap between rodents and humans. Like some rodent models and certain forms of the human Pelizaeus-Merzbacher and X-linked spastic paraplegia diseases, it arises from an exonic point mutation in the proteolipid protein gene (Nadon et al., 1990). The mutation arose spontaneously in Welsh springer spaniels and causes severe tremor from about 12 days of age after birth, followed by late onset convulsions (Griffiths et al., 1981; Duncan, 1995). The central nervous system (CNS) is

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severely hypomyelinated, with reduced numbers of mature oligodendrocytes (Duncan et al., 1983). Death normally occurs at about 3–4 months of age, but animals can live over 2 years with intensive rearing.

Xenografts of normal canine oligodendrocytes myelinate axons in myelin-deficient (*md*) rats (Archer et al., 1994), and allografting of canine glia into *sh* pup spinal cords shows that *sh* pup axons can be remyelinated (Archer et al., 1997). Allografting reduces immunologic complications but raises the problem of obtaining sufficient donor cells for transplantation, a common difficulty with species other than rodents. A solution may lie in the use of self-renewing, multipotential, growth factor–responsive neural progenitor cell populations, which grow in clusters dubbed "neurospheres" (Reynolds and Weiss, 1992, 1996; Reynolds et al., 1992). Cells in these populations generate neurons and astrocytes (Reynolds and Weiss, 1992, 1996; Reynolds et al., 1992) and oligodendroglial cells (Hammang et al., 1997; Reynolds and Weiss, 1996) in proportions that may be influenced by environmental manipulation. Murine neurospheres grafted into the *md* rat CNS generate cells that myelinate host axons (Hammang et al., 1994). Expandable multipotential populations of growth factor–responsive cells from the canine CNS could circumvent limitations of tissue availability for transplantation studies or in vitro analysis.

Although the presence of normal proteolipid protein or normal myelin production can distinguish normal from mutant oligodendrocytes after transplantation, both approaches are susceptible to technical or interpretational ambiguities. Recent glial transplantation has used *lac* Z expressing cells, obtained by stable transfection of rodent glial lines (Tontsch et al., 1994; Franklin et al., 1995, 1996) or from transgenic mice expressing *lacZ* under myelin protein promoters (Hammang et al., 1994; our unpublished data). Currently, no canine glial lines exist and neither approach can readily be applied in this species. Instead, we have tested the ability of the third generation PG13 retroviral vector system (Miller et al., 1991) to transduce *lacZ* expression in canine cells. Vector packaging in this system uses the Gibbon ape leukemia virus *env* protein, which includes both canine and human cells in its host range (Miller et al., 1991). A receptor, GLVR1, for this *env* protein is expressed abundantly in brain, with particularly high expression early in embryogenesis (Johann et al., 1992; Kavanaugh et al., 1994), making this system an ideal candidate for labeling canine neural progenitor cells.

We have obtained expandable multipotential populations of growth factor–responsive cells from the normal canine CNS and transduced these to express *lacZ* to provide labeled allogeneic donor cells for transplantation studies in *sh* pups or for xenografting into *md* rat recipients. We have also obtained similar populations from mutant *sh* pup brain, which should enable further study of the effects of the *sh* mutation on CNS cells.

MATERIALS AND METHODS

Preparation of "Neurospheres" From *sh* and *wt* Littermate Pups

Wildtype (*wt*) and *sh* pups were obtained from a colony at the University of Wisconsin. Donors were aged from embryonic day 40 to postnatal day 8. After euthanasia with barbiturate solution, brains were placed in artificial high-Ca^{2+}, low-Mg^{2+} cerebrospinal fluid solution (2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 124 mM NaCl, 5 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, pH 7.35) and the subventricular caudate nucleus, internal capsule, putamen/pallidum, and ventral mesencephalon separated and minced (<1 mm^3). Aliquots were dispersed mechanically by trituration or with enzymes (Reynolds and Weiss, 1992). All trituration was performed first with standard bore, then with fire-polished Pasteur pipettes, with the exception of trituration performed before transplant into *md* rats (see below), which used sequential passage through 20-, 23-, 25-, and 27-gauge needles. After filtration (35 μm nylon mesh; Small Parts, Miami Lakes, FL), suspensions were centrifuged (5°C, 5 min, 400*g*), dispersed in EGF^+ medium, comprised of 20 ng/ml mouse submaxillary gland EGF (Collaborative Research, Bedford, MA) in Dulbecco's Modified Eagle's Medium/F12 (1:1) with additives as given elsewhere (Reynolds et al., 1992), and plated in uncoated tissue culture flasks. To obtain adherent differentiated populations, cells were collected by centrifugation (5°C, 5 min, 400*g*), resuspended in EGF^- medium (the same base medium but with 1% fetal bovine serum replacing EGF) and plated on dishes coated with poly-L-ornithine (10 μg/ml).

Immunofluorescence

Rabbit polyclonal antibodies were used to detect nestin (Rabbit 130 from Dr. R. McKay, National Institute of Neurological Disorders and Stroke, Bethesda, MD) and 68 kD neurofilament protein (from Dr. P. Gambetti, Institute of Pathology, Case Western Reserve University School of Medicine, Cleveland OH). Monoclonal antibodies were Rat 401 anti-nestin (Developmental Studies Hybridoma Bank, Iowa City, IA), O4 and O1 (from Dr. M. Schachner), and Ranscht anti-galactocerebroside (GC) (Boehringer Mannheim, Indianapolis, IN). All other antibodies, conjugates, and nonspecific immunoglobulins were from Jackson Immunoresearch Laboratories (West Grove, PA). Negative controls were provided by omission of primary antibody or replacement with nonimmune rabbit serum or nonspecific mouse immunoglobulin. Secondary antibodies were the fluorescein-conjugates

goat anti-rabbit IgG and anti-mouse IgG, rhodamine-conjugated goat anti-mouse IgM, and biotin-SP-conjugated donkey anti-rabbit IgG and 7-amino-4-methylcoumarin-3-acetate-conjugated streptavidin. Cells were fixed with 4% paraformaldehyde (10 min, room temperature). Antibodies were diluted in 5% normal goat serum in phosphate buffered saline (with 0.1% Triton X-100 for antibodies to nestin). Aside from anti-GFAP (4°C, overnight), antibodies were incubated 1–2 hr at room temperature, followed by at least three buffer washes. After surface antigen labeling (O4, O1, Ranscht), samples were incubated in 5% glacial acetic acid:95% ethanol (v:v) for 10 min at −20°C and nestin, neurofilament, and glial fibrillary acidic protein (GFAP) antibodies used as above. Staining for combinations of O4, Ranscht and GFAP was as described elsewhere (Armstrong et al., 1992). Samples were mounted in CitiFluor (UKC Chemical Laboratory, Canterbury, UK) containing bisbenzimide H33342 fluorochrome (Calbiochem, La Jolla, CA).

Transduction of *lacZ* Expression in Canine Neurospheres

The PG13 retroviral packaging system, provided by Dr. M. Eiden, National Institute of Mental Health (Bethesda, MD), was maintained and used as previously described (Miller et al., 1991, 1993). Media conditioned for 6 days with packaged, defective, retroviral vector was harvested, filtered (0.45 μm), and used directly or stored at −70°C. Activity was confirmed using the Rat2 fibroblast line (Miller et al., 1991, 1993). Titers were in the order of 10^5 colony forming units per milliliter. Neurospheres were passaged (with trituration) into media consisting of EGF^+ medium:PG13-conditioned medium (1:1), with polybrene (4 μg/ml). After 20–24-hr incubation, cells were pelleted (5°C, 5 min, 400*g*), resuspended in EGF^+ medium, and collected 24–48 hr later by centrifugation (5°C, 5 min, 400*g*) for transplantation (below) or plating in EGF^- medium on poly-L-ornithine coated dishes for immunocytochemistry as above or 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal) staining (Tontsch et al., 1994).

Transplantation of Canine Neurospheres

After centrifugation as above, cells were resuspended in EGF^+ medium and either pipette triturated, followed by removal of undissociated spheres by nylon mesh filtration (35 μm), or triturated with needles as above. After Trypan Blue viability assessment and counting, cells were pelleted as above, resuspended at 25,000–75,000 cells/μl in Ca/Mg-free Hank's buffered saline solution with 0.01% bovine serum albumin and placed on ice until transplantation into *sh* pups.

Transplantation of Canine Neurospheres Into *sh* Pup Recipients

Recipient pups (aged either 14 days or 7 months) were premedicated with analgesics and sedatives, induced with an ultra-short-acting barbiturate, then intubated and maintained with isoflurane and oxygen. Dorsal laminectomy was performed at thoracic and lumbar sites T13–L1, L1–L2, and L2–L3. Using a surgical microscope, a durotomy was carried out at each laminectomy site. A glass micropipette (30-μm bore) was inserted into the spinal cord and 2–4 μl of cell suspension slowly injected over a 1-minute period by using a micromanipulator and Hamilton syringe. Respiration was controlled with the neuromuscular blocking agent succinylcholine during the injection to minimize spine movement. Injection sites were marked with sterile charcoal, a fat graft placed in each laminectomy defect and muscle, subcutaneous tissue and skin reapposed. Two to six weeks after injection, pups were anesthetized, fixative-perfused, and stained for X-gal, then transverse slices were Epon embedded for 1-μm sectioning and staining with *p*-phenylenediamine (Tontsch et al., 1994).

Transplantation of Canine Neurospheres Into *md* Rat Recipients

Cells were exposed three times to retroviral vector-containing medium that had been stored at −70°C (one freeze-thaw cycle). This boosted the transduction efficiency obtained with stored media. Initial overnight vector exposure, centrifugation, and resuspension in EGF^+ medium were as described above; after 48 hr, cells were again passaged with trituration into EGF^+ medium: PG13-conditioned medium (1:1) with polybrene (4 μg/ml), incubated 4 hr, then centrifuged and resuspended as before. This was repeated 72 hr later. Cells were passaged with trituration 48 hr later, and after a further 24 hr, triturated as above, except that the last trituration used 25½- and 27-gauge needles sequentially, and placed on ice for transplantation. The *md* rat recipients were from a colony at the University of Wisconsin. Transplantation into *md* rat spinal cord consisted of either one or two injections, the latter 1 mm apart, each of 1 μl at 25–50,000 cells/μl at the thoracic–lumbar T13–L1 junction, using published protocols (Hammang et al., 1994). Recipients were treated daily with Cyclosporine A (10 mg/kg body weight). Conditions of final anesthesia, tissue fixation, processing for X-gal revelation, Epon embedding, 1-μm transverse sectioning, and *p*-phenylenediamine staining have been described (Hammang et al., 1994; Tontsch et al., 1994).

RESULTS

Preparation of Neurospheres From *sh* Pup and *wt* Littermate Pups

We tested various tissue dissociation techniques on several regions from embryonic or postnatal *wt* canine brains. Donors were aged between embryonic day 40 to postnatal day 2 (canine gestation is about 63 days). Cells from embryos multiply more rapidly and have been expanded in vitro at least 6 months.

Neurosphere-like clusters were derived from each of the subventricular caudate nucleus, internal capsule, putamen/pallidum, and ventral mesencephalon. For all protocols, the putamen/pallidum was consistently the poorest source, both in cluster yields and in long-term sustainability of cultures. Subventricular and ventral mesencephalon regions were the richest sources of neurospheres from embryonic and postnatal pups and the most amenable to sustained in vitro expansion, as is also the case in both embryonic and adult mice (Reynolds and Weiss, 1992; Reynolds et al., 1992; Hammang et al., 1994, 1997). Separate canine preparations were subsequently made from (1) ventral mesencephalon and (2) "striatum," comprised of pooled caudate nucleus and the adjacent portion of the inner capsule.

Rodent neurospheres are obtained from CNS by trituration alone or after enzyme treatment (Reynolds and Weiss, 1992; Reynolds et al., 1992). Trituration alone routinely generated neurospheres from canine CNS, but enzyme treatment failed to give useful yields from some pups and subsequently was not used. Moreover, compared with rodent neurospheres, even after isolation, canine neurospheres were harder to dissociate during subsequent passaging or for differentiation studies. Even forceful trituration failed to disperse all clusters and, gauged by Trypan Blue exclusion, damaged as many as 80% of cells (data not shown).

In the murine neurosphere system, when cells are first isolated from the CNS there is a period of cell death over the first 5 days in vitro, followed by progenitor division leading to sphere formation (Reynolds et al., 1992). This was not observed in the canine system. Instead, adherent spherical clusters of refractile cells of healthy appearance were first detectable at 4 hr postplating and were readily apparent by 12 hr in both striatal and ventral mesencephalic cultures. As preparations were filtered before plating, these clusters may arise from cell aggregation. Nonadherent spherical clusters containing four or more refractile cells were observed within 12–72 hr postplating in essentially all cultures (Fig. 1A.). Debris and unhealthy or dead-looking cells were typically present during the first 14 days, but apparently healthy spheres of increasing size continued to be observed throughout this time. Dispersion of large clusters during passaging (see Methods and previous paragraph) yielded both small clusters and individual cells, with subsequent sphere growth allowing expansion at a split ratio of 1:2 every 2–4 weeks for at least 6 months

After expansion by passaging between two to nine times in the presence of EGF, adherent differentiated populations were obtained by partially dissociating neurospheres by trituration and plating onto poly-L-ornithine substrata in EGF medium. (As noted above, spheres could not be completely dissociated without excessive cell destruction.) Cells were fixed 1 hr to 28 days later. By 1 hr postplating, spheres had attached to the substratum. By 24 hr, emergent cells began to form monolayer "halos" around spheres.

Rat401 monoclonal and rabbit polyclonal 130 antibodies were used to reveal expression of nestin, a neural progenitor marker, in striatal or ventral mesencephalon-derived neurospheres. At 1, 4, and 24 hr postplating, fluorescence was above background levels in most cells (Fig. 1B), although labeling intensity with either antibody was low compared with staining seen with Rat401 in murine systems (Hammang et al., 1994; our unpublished observations), suggesting epitopic differences between canine and rodent nestin species. Both cells in clusters and some cells in the monolayer expressed nestin, but by 24 hr postplating many cells in the monolayer were apparently unlabeled (Fig. 1B) and no nestin staining was detectable after 10 days in vitro (not shown). Patterns of nestin staining were similar in cells originally derived from different CNS regions.

Monoclonal antibody to the 68 kD NF protein detected cells with morphologies resembling neurons or immature neuronal cells at 25 days postplating in both striatal and ventral mesencephalon-derived cultures (Fig. 1C). As revealed by this antibody, many of these cells had long, very fine processes. No cells expressing the 68-kD NF protein were detected at earlier times. Cells expressing GFAP, some with stellate morphology, were detected at all times examined from 7 days postplating onward in both striatal and ventral mesencephalon-derived cultures (Fig. 1D).

Markers used with or without concomitant GFAP staining to identify oligodendrocyte-type 2 astrocyte lineage cells were the O4 and Ranscht antigens (Sommer and Schachner, 1981; Ranscht et al., 1982). Process-bearing cells expressing O4 were detected within and surrounding spheres at all times examined from 7 days postplating onward in both striatal and ventral mesencephalon-derived cultures (Fig. 1E). Some, but not all, of these O4+ cells also expressed GFAP. Neither cells of stellate morphology nor process-bearing cells with morphologies typical of O4+ cells were observed in freshly

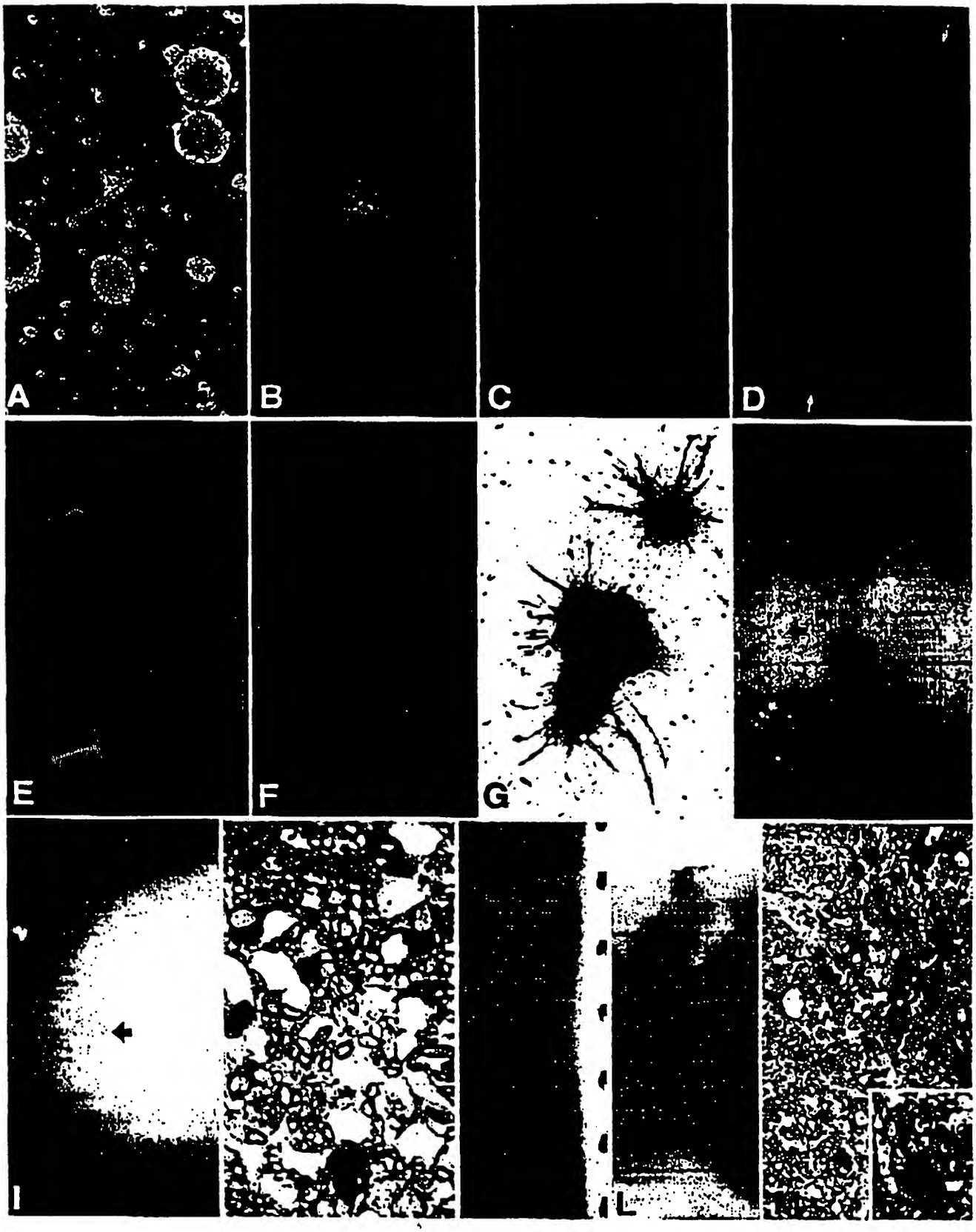

Figure 1.

plated neurosphere cultures nor in the surrounding halos of cells migrating out of the neurospheres in the first 24 hours after plating. Ranscht antigen expression was not detected at either 7 or 10 days postplating in any cultures but was observed at 12 days postplating in all cultures in a very small percentage of $O4^+$ cells (estimated to be $\ll 1\%$). These typically had morphologies classically associated with oligodendrocytes, with many highly branched processes (Fig. 1F). Such morphologies were not observed in freshly plated neurosphere cultures nor in the surrounding halos of cells migrating out of the neurospheres in the first 24 hr after plating from either earlier or later passages. These morphologic observations and patterns of marker expression, in particular the delayed and separate appearances of the Ranscht antigen and the 68-kD NF protein, provide direct evidence that differentiation along CNS lineages is occurring in this system.

The PG13 packaging system uses the Gibbon ape leukemia virus *env* protein (Miller et al., 1991). The retroviral vector we used in this system is based on the plasmid pLXSN and contains *lacZ* under the SV40 promoter (Miller et al., 1993). This vector/packaging combination transduced *lacZ* expression in a substantial proportion of cells in the canine neurosphere preparations generated from perinatal pups (Fig. 1G). We first used allografting into *sh* pups to address the issue of whether neurosphere-derived cells can survive in the adult *sh* pup CNS environment. Adult *sh* pups are extremely rare, and although the lifespan of *sh* pups can be increased to more than 2 years with intensive rearing, individual adults may nonetheless die at any time after 3–4 months even without the added risks arising from surgery. For these reasons, in this initial study, survival in an adult animal was examined in the short term, and a postnatal animal was used to examine survival in the longer term. Fixation and processing conditions for combined *lacZ* and myelin detection have been refined in numerous rat studies and are not optimal with respect to tissue preservation in the larger canine system, where animal rarity restricts optimization. Thus, the myelinating capacity of neurosphere populations was examined by xenografting into *md* rats, which also provides more stringent environmental conditions than allografting. Aliquots of populations destined for allografts or xenografts were plated under differentiating conditions, as above and in Methods, for parallel examination of in vitro differentiation and *lacZ* expression. The appearance of differentiation markers in these cultures followed the same time course as was described above.

Populations of proliferative canine neurospheres, originally derived from striatum of embryonic day 40–50 normal pups, were transduced to express *lac* Z and transplanted into the spinal cords of a postnatal 14 day and an adult (7 months) *sh* pup. (Donor cells had undergone three and nine passages in vitro, respectively, before transplant.) The postnatal pup was killed at 6 weeks and the adult pup at 2 weeks posttransplant. After X-gal staining, transverse sections of the spinal cord in the regions of the thoracic and lumbar injection sites (T13–L1, L1–L2, and L2–L3) revealed distinct clusters of blue patches in each of the postnatal and adult animals not only at the site of implantation in the dorsal column but also in the lateral or ventral columns and gray matter (Fig. 1H, I), suggesting that migration of implanted cells

Fig. 1. Generation and transplantation of EGF-responsive canine neural progenitor populations. A: Preparation from the striatum of a normal E45–50 pup at 48 hr after isolation from the CNS, showing spherical clusters resembling neurospheres containing refractile cells of healthy appearance. B: Nestin immunoreactivity (green) occurs in cells within and around spheres in normal E50 canine striatal neurosphere preparations 24 hr after plating in EGF medium. Some surrounding cells, visualized by nuclear labeling with Hoechst bisbenzimide H33342 (blue), do not express nestin. C: Cells from normal E50 canine striatal neurospheres 25 days after plating in EGF medium, labeled for the 68-kD neurofilament protein (green) and Hoechst bisbenzimide H33342 (blue). D: Cells from normal E50 canine striatal neurospheres with stellate and other morphologies expressing GFAP (blue) 7 days after plating in EGF^- medium. Nuclei are also labeled blue with Hoechst 33342, which does not label cell processes, allowing GFAP-negative cells to be visualized (arrows). E: Expression of O4 antigen (red) in cells from normal E50 canine ventral mesencephalon neurospheres 12 days after plating in EGF^- medium. F: Occasional O4-positive cells also react with the Ranscht anti-galactocerebroside antibody at this time. G: Expression of *lacZ* in normal canine neurospheres after transduction using the PG13 retroviral vector system. H: Transverse section of the spinal cord of a 2-month *sh* pup, 6 weeks after transplantation of *lac* Z-labeled *wt* canine neurospheres at 14 days after birth. Blue clusters are visible in several regions of the cord (arrows). I: Transverse section of the spinal cord of a 7–8-month *sh* pup, 2 weeks after transplantation of *lacZ*-labeled *wt* canine neurospheres. A blue cluster is visible in the lateral column of the spinal cord (arrow). J: Microscopy of transverse sections shows graft cells apparently integrated normally into the adult *sh* pup cytoarchitecture (arrows). Vacuolation in this section is due to the method of fixation and not the transplanted cells. The section is counterstained with paraphenylenediamine. K: Blue clusters are visible for several millimeters along the dorsal midline of the spinal cord of an *md* rat that received *lacZ*-labeled canine neurospheres 1 week after birth (1-mm markers on right). L: The same spinal cord shown in K, showing blue labeling throughout the dorsal column at the site of injection. M: In a 1-μm section from the spinal cord shown in L, scattered myelinated fibers (more than seen in nontransplanted *md* rats) are present. Details of a normal oligodendrocyte with a long cytoplasmic process extending to a myelinated axon (arrow) are shown in the inset. Such cells are not normally seen in areas outside of the transplant.

may have occurred. Our previous studies have shown that there is an acute passive dispersion of cells only along the dorsal column, up to 8 mm from the site of transplantation (Lipsitz et al., 1995). Similar studies with Hoechst labeled cells have also shown a localization of transplanted cells immediately after transplant to the dorsal columns (Zhang and Duncan, unpublished data). The fixation used failed to preserve structures optimally in the adult mutant, but nonetheless light microscopy of tissue sections clearly demonstrated *lacZ*-expressing cells in association with areas containing myelin (Fig. 1J). These cells appeared to have integrated into the surrounding cytoarchitecture, with no evidence of rejection.

Xenografts into *md* rat recipients were used to obtain further evidence for the ability of neurosphere-derived cells to myelinate host axons in a myelin-deficient environment. To facilitate routine studies in these rats, a triple exposure protocol was used for successful transduction of *lacZ* expression by using media conditioned with packaged, defective, retroviral vector that had been stored frozen before use (Methods). Canine neurospheres were transduced to express *lacZ* and implanted into the dorsal columns of spinal cords of 7-day postnatal *md* rats at the thoracic–lumbar (T13–L1) junction (Methods). At 11 days posttransplant, low-power light microscopy revealed blue X-gal reaction product spread up to 6 mm along the dorsal midline of at least three transplant recipients in each of two separate experiments (Fig. 1K). This typically appeared as clusters of small blue patches or dots, rather than the more homogeneously distributed streak of blue-labeled cells often seen after glial cell transplantation (Tontsch et al., 1994). Transverse sections (1 μm) of recipient spinal cords revealed grafted cells in the dorsal columns in areas containing myelinated axons (Fig. 1L) and, strikingly, a grafted cell with a long cytoplasmic process in contact with a myelinated axon (Fig. 1M). By electron microscopy (EM), the myelin was found to have a normal intraperiod line that is lacking in host myelin.

DISCUSSION

We have obtained cell populations from *wt* canine perinatal striatum and ventral mesencephalon that closely resemble murine EGF-responsive multipotential neural progenitor cell populations (neurospheres). The clusters of canine cells are similar in appearance to murine spheres and can be expanded with passaging for at least 6 months in vitro in the presence of EGF. On removal of EGF, cells within the clusters generate neurons, astrocytes, and oligodendroglia. Cells from these cultures can survive at least 6 weeks in postnatal and at least 2 weeks in adult *sh* pup recipients. This is the first time neurosphere transplant survival has been demonstrated in the adult CNS. Neurospheres thus provide a new source of allogeneic donor cells for transplantation studies in this mutant. Moreover, grafting of *wt* neurospheres into *md* recipient spinal cords resulted in the production of apparently normal myelin by graft-derived cells, showing that these cells are able to myelinate a myelin-deficient host. The ability of neurosphere-derived cells to myelinate under xenograft conditions is encouraging for future studies involving human neurospheres, which must first be tested by xenografting. In addition, the PG13 retroviral vector packaging system, based on the Gibbon ape leukemia virus *env* protein, provides a new means of labeling donor cells. With a host range also including humans and rodents, this system is likely to be useful for transplantation studies in various species.

Our observations of neurospheres within a few hours of the initial preparation of cultures from the CNS suggest rapid extensive reaggregation of cells. Early "spheres" are unlikely to arise from proliferation of single progenitors as canine neurospheres grow slowly compared with murine neurospheres maintained in identical media (our unpublished observations). Human fetal neurosphere preparations also grow very slowly compared with murine preparations and may require different combinations of factors for optimal growth (B. Reynolds, personal communication). Faster growth apparently occurred in preparations obtained from embryonic, as opposed to postnatal, canine CNS. Because initial yields from postnatal pups were in general lower than those obtained from embryos, the relative success of the latter may reflect either or both developmental differences in the proliferative capacities of progenitors or growth-enhancing autocrine effects, which may occur in higher density cultures.

Removal of EGF resulted in the sequential appearance, over a period of 3–4 weeks, of cells with distinctive differentiated morphologies expressing markers of mature astrocytes, oligodendrocytes, and neurons, which were not detected in freshly plated neurosphere preparations. Thus canine neurosphere-derived cells differentiate along at least two lineages, although we could not verify that these neurospheres do, in fact, contain multipotential cells rather than subpopulations of lineage-committed, unipotential progenitor cells, because the slow growth rate of these cells has prevented clonal analysis to date. Clonal analysis has confirmed the existence of self-replicating neural stem cells in analogous EGF-responsive murine neural progenitor populations (Reynolds and Weiss, 1996), and the long-term expandability of canine populations is indirect evidence that such cells may be present. However, whether or not stem cells are present, as the first expandable differentiating system established from canine brain, neurospheres provide a means to study at least three of the major CNS cell species, which has not

previously been available in the dog and which should facilitate studies both of normal canine and also of *sh* pup CNS.

Although secondary to the aims of the present study, we have had preliminary success in generating neurospheres from *sh* pup mutant embryos (data not shown), providing a new in vitro system for characterizing the effects of the mutation. The developmentally regulated alternative splicing pattern of the myelin proteolipid protein gene is altered in the *sh* mutant, suggesting delayed mutant oligodendrocyte maturation (Nadon et al., 1990). Proteolipid protein gene expression may also have functions in CNS development before myelinogenesis. The ability to generate neurospheres that are similar in many respects to those derived from *wt* brain should facilitate studies on differentiation of *sh* pup oligodendrocytes. Such *sh* pup neurospheres could also be used as a target in gene transfer studies. In addition, our success in expanding neurospheres from the *sh* pup mutant suggests that this approach could extend the range of models amenable to study and may be applicable to very rare mutants, in which limited availability of affected animals has restricted characterization to date.

Although expansion of the canine populations is slow compared with the murine case, the scarcity of source tissue in the canine system makes these cells a valuable alternative to primary culture. Analysis of growth factor effects in the canine system may permit faster expansion of these cells in vitro. Basic fibroblast growth factor (bFGF) stimulates proliferation of some rodent neural progenitor populations (Lillien and Cepko, 1992; Richards et al., 1992; Ray and Gage, 1994; Kilpatrick and Bartlett, 1995; Vescovi et al., 1993) and has been implicated in oligodendroglial proliferations and inhibition of differentiation or population reversion to more immature phenotypes in both rat (Bogler et al., 1990; McKinnon et al., 1990) and human models (Armstrong et al., 1992; Gogate et al., 1994). It could well have similar effects in the canine system, in which bFGF (5–100 ng/ml) may increase proliferation in canine oligodendroglial-enriched cultures (Hoffman and Duncan, 1995). Sequential growth factor mixtures could enhance first proliferation then differentiation of canine neural progenitor cells.

Myelin deficiency may activate compensatory mechanisms. Even when these are insufficient to override effects of endogenous myelin gene mutations, the environment may nonetheless express factors favoring oligodendrogenesis and myelinogenesis by transplanted cells. Neurospheres generated myelinogenic oligodendrocytes when transplanted into the *md* rat, and furthermore, grafted neurosphere-derived cells survived not only in the neonatal but also in the adult *sh* pup. Thus the adult canine environment is able to support the survival of perinatal neural progenitor cells, at least in the short term. This is consistent with the existence of neural progenitor cells in adult rodent CNS (Reynolds and Weiss, 1992; Richards et al., 1992). The number of myelinated axons observed after xenografting of canine neurospheres into the *md* rat was considerably more than is seen in nontransplanted *md* rats, although less has been seen with allografts of murine neurospheres, which myelinate large areas of the *md* cord (Hammang et al., in press). In view of the in vitro differences in expansion rates discussed above, canine neurospheres may also take longer to expand and mature in vivo than murine neurospheres, even though the presence of apparently myelinating transplant-derived oligodendrocytes after only 11 days in vivo suggest canine oligodendrocytes differentiate more rapidly in vivo than in vitro. This system should be valuable for future study of survival, growth, and myelinating ability of grafted cells, particularly in adult recipients, with ultimate applications in humans.

Unlike some more differentiated CNS cell species, neural progenitor cells may not express both classic histocompatibility molecules and may thus, at least initially, provoke a less intense host immune reaction (Bartlett et al., 1990). Although null mutant studies suggest peripheral transplant rejection can occur in the absence of both class I and II molecules, as far as we are aware this has yet to be established for the CNS, which is relatively immunologically privileged. Neurospheres also offer the prospect of generating mixed populations of neural cell species in a controllable manner. Because both astrocytes (Raff et al., 1985; Richardson et al., 1988) and neurons (Barres and Raff, 1993; Hardy and Reynolds, 1993) have been implicated in oligodendroglial migration and differentiation and may also influence myelination, this could benefit transplant-derived remyelination. As well as advantages of manipulability and in vitro expandability, these cells provide a multipotential, proliferation-competent pool from which differentiated populations may arise in response to environmental cues. Implantation of small donor populations could ultimately assist in maintaining lifelong reservoirs from which CNS cell populations may be renewed through endogenous or externally manipulated environmental signals.

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Adult brain retains the potential to generate oligodendroglial progenitors with extensive myelination capacity

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ABSTRACT **Remyelination of focal areas of the central nervous system (CNS) in animals can be achieved by transplantation of glial cells, yet the source of these cells in humans to similarly treat myelin disorders is limited at present to fetal tissue. Multipotent precursor cells are present in the CNS of adult as well as embryonic and neonatal animals and can differentiate into lineage-restricted progenitors such as oligodendroglial progenitors (OPs). The OPs present in adults have a different phenotype from those seen in earlier life, and their potential role in CNS repair remains unknown. To gain insights into the potential to manipulate the myelinating capacity of these precursor and/or progenitor cells, we generated a homogenous culture of OPs from neural precursor cells isolated from adult rat subependymal tissues. Phenotypic characterization indicated that these OPs resembled neonatal rather than adult OPs and produced robust myelin after transplantation. The ability to generate such cells from the adult brain therefore opens an avenue to explore the potential of these cells for repairing myelin disorders in adulthood.**

Remyelination of the central nervous system (CNS) in patients where host remyelination fails or where the endogenous myelinating cells are genetically impaired may be achieved, at least focally, by glial cell transplantation. It has been assumed that human fetal brain will be the only viable source of myelinating cells for human transplantation, because oligodendroglial progenitors (OPs) derived from embryonic animals have a greater capacity for myelination than mature cells after transplantation (1, 2). However, the availbility of human fetal tissues remains a practical and ethical concern, and it would be preferable if the neonatal or adult human brain could be used as a source of myelinating cells. It has been established that OPs are present in adult human brain (3). Such cells have also been described in patients with multiple sclerosis (4) and in rodents with chronic experimental allergic encephalomyelitis (5). Despite their presence in chronic multiple sclerosis lesions, remyelination may be inadequate (6, 7), and either exogenous myelinating cells must be targeted to lesions or host cells must be recruited to aid in repair.

Two types of OP [also designated *in vitro* as oligodendrocyte type-2 astrocyte (O2A) progenitor] exist in the CNS; the neonatal OP ($O2A^{perinatal}$) that appears in the rat postnatally and disappears about 6 weeks after birth, and the adult OP ($O2A^{adult}$) (8, 9). The $O2A^{adult}$, which is identified by the mAb O4 *in situ* and *in vitro*, has a phenotype that distinguishes it from its neonatal counterpart. The most thoroughly characterized $O2A^{adult}$ cells are those isolated from adult rat optic nerves, although similar cells are found in other parts of the CNS such as the spinal cord (10). Unlike the $O2A^{perinatal}$, the $O2A^{adult}$ does not express the intermediate filament vimentin or a ganglioside recognized by the mAb A2B5. The $O2A^{adult}$ cells also have a longer cell cycle time (65 ± 18 h) and are less motile (4 ± 1 μm/h) than $O2A^{perinatal}$ (9). These characteristics suggest that they would only have a limited capacity to remyelinate demyelinated areas of the brain. In fact, it is not yet known whether these cells produce myelin *in vivo*, for example, after transplantation.

The OPs are generally thought to be derived from multipotent neural precursor cells or early progenitor cells in the CNS. Neural stem cells, which can give rise to both neurons and glia, have been found in the CNS of both embryonic and mature animals (11, 12). Clonal analyses suggest that the stem cells from adult CNS are similar to those of embryonic origin (11). At least, these adult stem cells can differentiate into neurons, astrocytes, and oligodendrocytes *in vitro*. It is not yet known whether adult stem cells differentiate into $O2A^{adult}$ directly.

We have been studying the transition from multipotent precursor cells to lineage-restricted OPs and have shown that it is possible to generate a large number of self-renewing OPs from neural precursor cells derived from embryonic and neonatal brain (13, 14). Because multipotent stem cells exist in adult CNS, we sought to explore whether the OPs derived from adult neural stem or precursor cells have the capacity for extensive myelination. If this were proven in the rodents, a similar approach could provide cells for transplantation or suggest means for the induction of endogenous progenitors to enhance host repair in humans.

MATERIALS AND METHODS

Cell Culture. The neural precursor cells in suspension culture ("neurospheres") were prepared from subependymal striata of Wistar rats aged 3 and 16 months according to a protocol detailed previously (13, 15). The culture medium was DMEM/F-12 (1:1) supplemented with insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and sodium selenite (30 nM). The above medium, referred to as "neurosphere medium," was supplemented with 20 ng/ml human recombinant epidermal growth factor (EGF) or EGF plus 20 ng/ml of basic fibroblast growth factor (bFGF) (Collaborative Biomedical Products, Bedford, MA). In the initial week of culture, B27 (GIBCO) was added to the above medium. The cultures were incubated in a humidified atmosphere of 5% CO_2/95% air with a partial medium change every other day.

The B104 neuroblastoma cells were cultured according to Louis *et al.* (16), and the conditioned medium (B104CM) was collected and filtered after 3 days of conditioning the B104 cells with serum-free "neurosphere medium."

BrdUrd Incorporation Assay. The coverslip cultures were incubated in 10 μM BrdUrd (Sigma) for various periods (see

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Abbreviations: CNS, central nervous system; OP, oligodendroglial progenitors; O2A, oligodendrocyte type 2 progenitor; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein; PLP, proteolipid; DIV, days *in vitro*.

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Results), fixed in acidic ethanol, and immunostained with anti-BrdUrd antibody (Amersham Pharmacia) at a dilution of 1:10, followed by fluorescein-labeled secondary antibody. For cell cycle time estimation, the cultures were exposed to BrdUrd for a period of 0.5, 1, 3, 5 and up to 16–24 h. The BrdUrd-labeled cells and the total cells stained with Hoechst were counted under a fluorescent microscope. The percentage of the labeled cells was plotted against the time the cells were pulsed, and the cell cycle time was estimated according to the graphic method of Sasaki *et al.* (17).

Assay of Cell Migration. A single sphere was plated onto ornithine-coated 35-mm dishes in a drop of medium. After the sphere attached (10–15 min), 1.5 ml of medium was added gently. Only the samples with successfully attached sphere and without floating cells were followed at 4, 8, and 24 h postplating. The outgrowth of the sphere was examined under the phase-contrast microscope, and the images were photographed and stored in a computer. The longest distance from the edge of a sphere to the cell body in each quarter of the outgrowth was measured and the average distance of cells moved out of a single sphere at specific time points was calculated (13). At least 8 spheres were followed throughout the period of each individual experiment, and the experiment was repeated twice.

Immunocytochemistry. Free-floating spheres or coverslip cultures were immunolabeled with fluorescein-tagged secondary antibodies (Jackson ImmunoResearch) according to the procedure detailed previously (13). The following primary antibodies were used. Monoclonal antibody anti-nestin (IgG) was a supernatant of mouse hybridoma rat401 (diluted 1:5), provided by Developmental Studies Hybridoma Bank (The Johns Hopkins University, Baltimore). A2B5 was a culture medium of mouse hybridoma clone 105 (American Type Culture Collection, CRL-1520, used at 1:100 dilution). O4 and O1 (both were IgM) were provided by M. Schachner. Anti-myelin basic protein (MBP, mouse IgG, 1:100) was from Boehringer Mannheim. Anti-vimentin (mouse IgG) and anti-β_{III}-tubulin (rabbit IgG) were purchased from Sigma (1:100). Polyclonal antibodies anti-glial fibrillary acidic protein (GFAP, 1:200) was purchased from Dako, and anti-platelet-derived growth factor receptor α (PDGFRα, 1:100) was from Santa Cruz Biotechnology.

Transplantation of Oligosphere Cells. The oligospheres were triturated into single cells and were then concentrated to 50,000 cells per microliter. One microliter of cell suspension was transplanted into the spinal cord of postnatal day 6–8 myelin-deficient (*md*) rats according to the procedure described (13, 18). The injection site was marked with sterile charcoal before the incision was sutured.

Twelve to fourteen days after transplantation, the recipient rats were anesthetized with pentobarbital (i.p.) and perfused with 4% formaldehyde. The spinal cord was dissected and the white streak representing myelin made by the transplanted cells was measured. The spinal cords were then trimmed for immunostaining with anti-proteolipid protein (PLP, a gift from I. R. Griffiths, University of Glasgow) or for resin-embedding as described (13, 14).

RESULTS

Establishment of OP Cultures from Adult Rats. The OPs were generated from neural precursors by using the approach described (13, 14). In the present study, cultures of neurospheres were initiated from subependymal striata of adult Wistar rats (aged 3 and 16 months). When cultured in the presence of EGF and absence of substrate, scattered phase-bright cells were found among debris at 4–7 days *in vitro* (DIV). These few cells grew into spheres in the subsequent 2–3 weeks. These spheres were triturated into single cells and expanded in the presence of EGF alone or EGF plus bFGF. Expanded neurosphere cells were immunopositive for nestin (Fig. 1 *a* and *b*), an intermediate filament protein mainly expressed by stem or precursor cells (19). When plated on poly(ornithine)-coated coverslips in the presence of 1% FBS but the absence of EGF or bFGF, the neurosphere cells migrated out and differentiated into a mixture of mainly astroglia (GFAP+) with flattened cell bodies and thick processes and some oligodendroglia (O4+). Some spheres also contained neurons that were β_{III}-tubulin+ (data not shown). The neurospheres were triturated into single cells and passaged in neurosphere medium with the presence of EGF and bFGF. These observations suggest that neurosphere cells are undifferentiated neural precursor cells, similar to those isolated from embryonic and neonatal striatum (13, 14).

To generate OPs from neurospheres, we gradually changed the EGF-containing medium to B104CM-containing medium by replacing one-fourth of the former medium with the latter medium every other day. During the transition period (1–2 weeks), the number and size of spheres did not increase. This is similar to the phenomenon observed in the neurosphere cultures from neonatal rat (13). By week two, the size and number of spheres began to increase. Three to four weeks later, the cultures were passaged in medium containing B104CM (30%) but no EGF or bFGF by plating 1×10^6 cells into a 75-cm^2 flask. New spheres with various sizes formed in

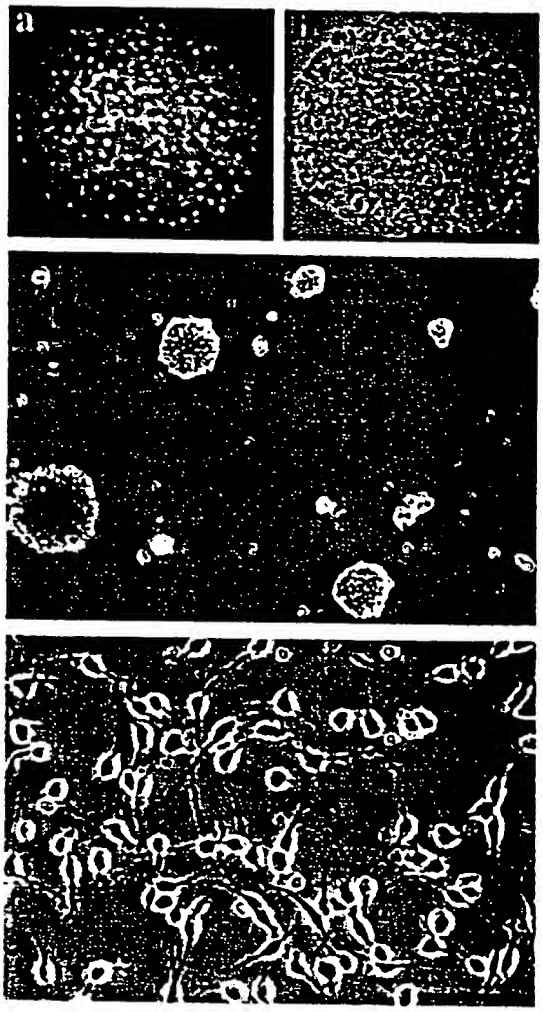

FIG. 1. A neurosphere (from 16-month-old rat) grown in the presence of EGF immunostained with nestin indicated that all cells were nestin+ (*a*). *b* shows the phase-contrast image of *a*. (*c*) New spheres were generated from disaggregated oligosphere cells. (*d*) Disaggregated oligosphere cells displayed bipolar or tripolar morphology in the presence of B104CM. (Bar = 100 μm.)

1 week (Fig. 1*c*). When the spheres were triturated into single cells and plated onto ornithine-coated coverslips, all cells displayed bipolar or tripolar morphology, typical of O2A progenitors (Fig. 1*d*). Therefore, the spheres were now referred to as "oligospheres," a term that was first used by Evercooren and colleagues (24). Similar results were obtained when generating oligospheres from neurospheres that were derived from both 3-month- and 16-month-old rat brains by using the same protocol.

Antigenic Expression of Oligosphere Cells. The $O2A^{perinatal}$ displays a bipolar morphology and is positive for A2B5, whereas the $O2A^{adult}$ is unipolar and O4+ (9). In contrast to the $O2A^{adult}$ previously derived from the adult optic nerve, all oligosphere cells exhibited bi- or tripolar morphology and expressed vimentin, A2B5, and PDGFRα (Fig. 2*a–c*) when the oligospheres were disaggregated and cultured on ornithine-coated coverslips at a density of 1×10^5 per coverslip in the presence of B104CM. These cells were negative for O4 (Fig. 2*d*). Within a week, the cultures were confluent. Similar results were obtained when the cells were cultured in the presence of both PDGF (10 ng/ml) and bFGF (20 ng/ml) except that they did not reach confluency until about 10 DIV. When the cells were cultured in the presence of PDGF alone with addition of PDGF every other day for 7 DIV, many cells were still bipolar or tripolar (Fig. 2*e*) and the majority were positive for A2B5 ($90.9 \pm 2.4\%$; $n = 5$), vimentin, and PDGFRα. A small number of cells ($5.2 \pm 3.0\%$; $n = 5$), however, became multiprocess-bearing and O4+. In addition, some cells were round without processes. These round cells were positive for A2B5 and vimentin but negative for O4, similar to those seen in the presence of B104CM.

Differentiation of Oligosphere Cells. The $O2A^{adult}$ cells differentiate more slowly than their neonatal counterparts (9). To assess the potential and speed of differentiation, oligosphere cells were cultured in the medium consisting of DMEM and 0.5% FBS. The cultures were immunostained with O4, O1,

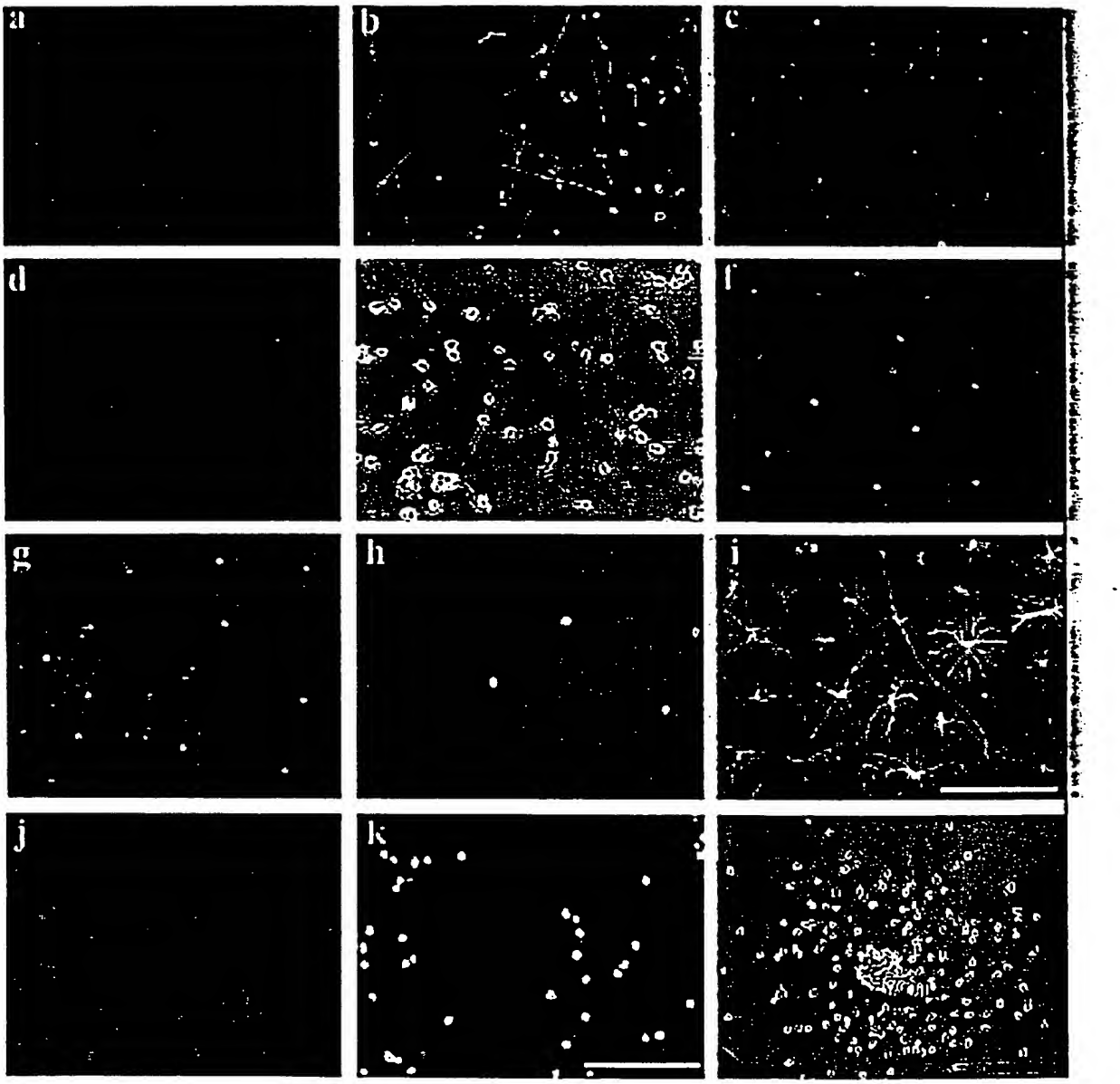

FIG. 2. The oligosphere cells cultured on ornithine-coated coverslips in the presence of B104CM were positive for vimentin (*a*), A2B5 (*b*), and PDGFRα (*c*) but negative for O4 (*d*). In the presence of PDGF alone for 7 DIV, the oligosphere cells were largely bipolar or tripolar. There were also round cells (arrowheads) and a few multiprocess-bearing cells (arrows) (*e*). In the presence of 0.5% FBS for 2 DIV, all cells were O4+ (*f*) and many cells were O1+ (*g*). At 7 DIV, cells were MBP+ (*h*). In the presence of 10% FBS, almost all cells were positive for GFAP (*i*) and A2B5+ (*j*). Incubation of the culture with BrdUrd for 20 h indicated that the majority of cells were labeled in the nuclei (yellow in *k*). All cells were A2B5+ (red in *k*). (*l*) A single sphere plated on ornithine-coated dish in the presence of B104CM for 24 h shows that bipolar cells migrated out of the sphere. The nuclei of cells in (*f–h*) were stained with 4',6-diamidino-2-phenylindole (DAPI). (Bar = 100 μm.)

and anti-MBP antibodies, which recognize progressively later developmental stages of oligodendroglial lineage. At 2 DIV, virtually all of the cells were O4 + (Fig. 2*f*). At the same time, 57.5 ± 4.4% (n = 6) of the cells were already O1 +, although the staining was mainly in the cell bodies and main processes (Fig. 2*g*). At 3 DIV, the majority of cells were O1+. At 5–7 DIV, most cells were positive for MBP, displaying membrane-like structures (Fig. 2*h*). In the presence of high concentrations of FBS (5–10%), the majority of cells were flattened, with star-shaped processes, and expressed both GFAP (Fig. 2*i*) and A2B5 (Fig. 2*j*). Similar results were obtained when oligosphere cells of passage 4 or 12 from both ages were examined.

Proliferation Potential. When 1×10^6 oligosphere cells were plated in the presence of 30% B104CM, $(8.8 \pm 1.2) \times 10^6$ (n = 3) cells were obtained in 7 DIV. A similar number of cells were generated when oligospheres from passage 2–12 were examined. The oligospheres could also be expanded in the presence of PDGF plus bFGF, although the yield was lower.

The cell cycle time for oligosphere cells in the presence of B104CM was estimated by using the graphic method described by Sasaki (17). The phase of DNA synthesis was deduced as 6.8–8.4 h from the linear regression of BrdUrd incorporation over incubation time (based on three independent experiments). The total cell cycle time was estimated to be about 20 hours. Incubation of the cells with BrdUrd for 20 h led to ≈92% of the cells labeled with anti-BrdUrd (Fig. 2*k*).

To assess the proliferation potential of oligosphere cells in response to growth factors, oligospheres (passage 4 and 10) were triturated and cultured for 3 days on coated coverslips in the presence of B104CM (30% vol/vol), bFGF (20 ng/ml), PDGF (10 ng/ml), and PDGF plus bFGF. The cultures were then exposed to BrdUrd for 4 h, and the incorporation of BrdUrd into nuclei was assessed. Without the presence of B104CM or above growth factors, cells differentiated into oligodendrocytes (O1+) and did not incorporate BrdUrd. In the presence of B104CM or growth factors, cells incorporated BrdUrd into their nuclei. The highest percentage of cells incorporating BrdUrd were the cells treated with B104CM (46%), followed by bFGF plus PDGF, bFGF, and PDGF (Table 1). This pattern of growth response of oligosphere cells is similar to that of $O2A^{perinatal}$ cells in response to growth factors (20, 21).

To examine whether a single cell can renew itself and regenerate an oligosphere, a single sphere cell was plated in each well of a 96-well plate containing 200 μl of B104CM (30%)-containing neurosphere medium (13). After 7 days, the plates were reexamined, and the wells containing sphere(s) were marked. The percentage of the cells able to generate new sphere(s) was ~29% (32/111). The clonally expanded cells retained the same potential to differentiate into oligodendroglia or type-2 astroglia *in vitro* (see above). Similar results were obtained when a single cell was plated into ornithine-coated 96-well plate except that the generated cells did not form a sphere (data not shown).

Migration of Oligosphere Cells. After the oligosphere attached, individual cells migrated out of the sphere within 1 h. At 4 h post-plating, cells were found surrounding the whole sphere. The migration velocity was calculated based on the average distance of cells moving away from the sphere at 4, 8, 12, and 24 hours post-plating. Migration velocity was 25 ± 5.4 μm/h (n = 10) in the presence of B104CM and 13.5 ± 1.7 μm/h (n = 8) in the presence of PDGF (10 ng/ml) for oligospheres (passage 8) derived from the 3-month-old rat. Cells migrating out of the sphere were bipolar (Fig. 2*l*). Unlike the oligosphere cells derived from neonatal rat, the pattern of migration was not always radially oriented. Similar results were obtained when spheres from the 16-month-old rat (passage 6) were examined.

Table 1. BrdUrd incorporation by oligosphere cells

	PDGF	bFGF	PDGF/bFGF	B104CM
BrdUrd+, %	29.5 ± 2.84	34.2 ± 2.27	36.9 ± 3.25	46 ± 4.13

BrdUrd+ cells and total cells were counted in four optic fields of each coverslip. Each group consisted of at least four coverslips. Total cell counts in each group were 3,300–3,965. The data were from the experiment with passage 4 cells derived from a 3-month-old rat.

Myelination Potential by Oligosphere Cells. Oligosphere cells of passage 8 from a 16-month-old rat and passage 4 and 12 derived from a 3-month-old rat were transplanted into the spinal cords of 24 *md* rats. Twelve to fourteen days after transplantation, a white streak, of average 4 mm (3.0–6.5 mm) in length, was present in the dorsal column of the spinal cord of the *md* rat, which is otherwise semitranslucent because of the lack of myelin (Fig. 3*a*). A white streak of 3.9 ± 1.25 mm (n = 7) formed by cells of passage 4 and 3.8 ± 1.5 mm (n = 8) formed by cells of passage 12 that were both derived from the 3-month-old rat. When cells (passage 8) from the 16-month-old rat were transplanted, a white streak of 4.2 ± 1.0 mm (n = 9) formed. There was no difference in the degree of longitudinal spread of transplanted cells and myelination by cells from both ages or cells from passage 4 and 12. A cross section of the spinal cord indicated that the white patch occupied most of the dorsal funiculus. Immunostaining of the spinal cord sections indicated that the myelin sheaths formed by the transplanted cells were positive for PLP (Fig. 3*b*) as well as for MBP (data not shown). The host spinal cord lacks PLP-positive myelin because of a mutation in the PLP gene (22), although PLP+ oligodendrocytes were detected in freshly prepared tissues (Fig. 3*b*). Toluidine blue-stained semithin sections (1 μm) confirmed that the majority of axons in the dorsal funiculus were myelinated (Fig. 3*c*). There was no obvious difference between the samples with cells from different ages in terms of the amount of myelin that are present in the transverse section.

DISCUSSION

The major finding of this study is that the adult brain can be used as a source of OPs with the $O2A^{perinatal}$ phenotype and that these cells can be propagated extensively to generate a large number of progenies that maintain their myelinating potential. If similar approaches were feasible in humans, it would be possible to generate large numbers of cells by *ex vivo* manipulation with growth factors, before transplantation. Similarly, it raises the possibility that such cells might be induced to expand by *in vivo* growth factor application and be recruited to target areas of demyelination in the human brain.

Oligosphere Cells Derived from Adult Brain Resemble $O2A^{perinatal}$ Cells. $O2A^{perinatal}$ cells can be isolated and expanded from neonatal rodents by using growth factors or conditioned media when the cells are in peak proliferation (23–25). We have explored alternative means of deriving such cells from multipotential neural precursor cells isolated from neonatal (13) or embryonic rat brains (S.-C.Z., unpublished data) by analogy to the hematopoietic cell lineage development (26). Because multipotential precursor cells exist in the CNS of adult (11, 15) as well as in embryonic stage (12), it is possible that OPs may be generated from adult CNS precursor cells as well. Therefore, the establishment of a homogeneous population of OPs from adult neural precursors was not unexpected. However, that all of the cells were positive for vimentin and A2B5 but negative for O4 contrasts with the antigenic phenotype of the $O2A^{adult}$ as isolated directly from adult rat optic nerves (9). More importantly, the oligosphere cells proliferate much more vigorously and differentiate and migrate faster than the $O2A^{adult}$ progenitors detailed in a series of studies performed by Noble and colleagues (9, 28–30). Therefore, the OPs from adult neural precursor cells resemble neonatal rather than adult O2As isolated directly

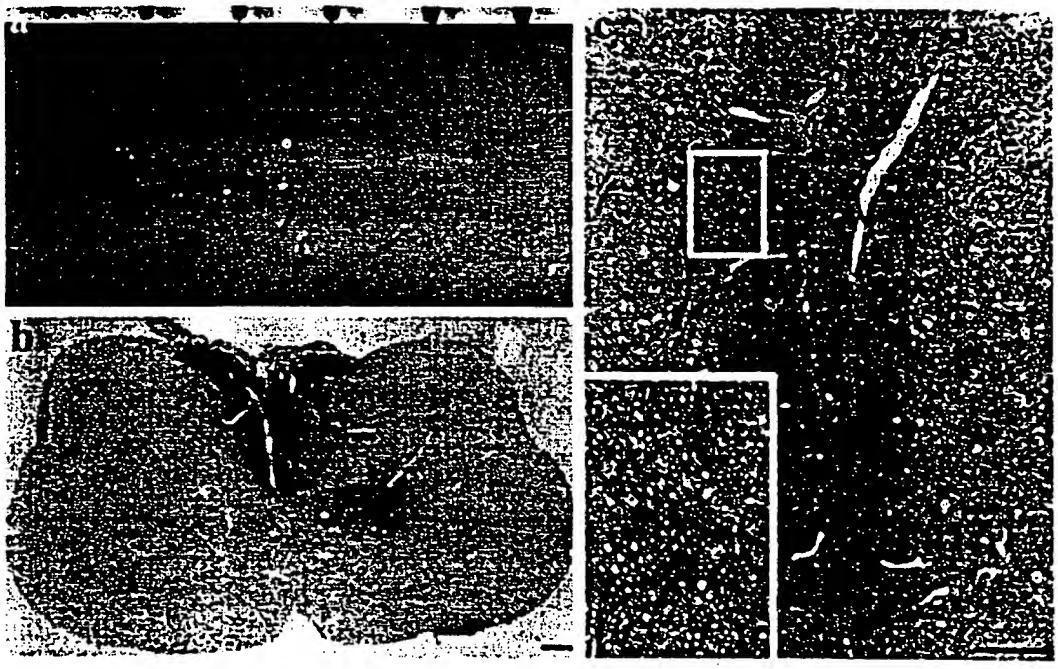

FIG. 3. Transplantation of oligosphere cells from a 16-month-old rat into *md* rats. Twelve to fourteen days later, a white streak of myelin was seen along the dorsal surface of the cord (*a*). The black dots are sterile charcoal marking the injection site. The space bar on top represents 1 mm. (*b*) Immunostaining of the transplanted cord showed PLP+ myelin in the dorsal funiculus with some myelin also appeared in the gray matter. Other areas of the spinal cord showed no PLP+ myelin except the PLP+ cell bodies. (*c*) Semithin sections stained with toluidine blue demonstrated that the dorsal funiculus was occupied by a large number of myelin sheaths. *Inset* is the enlargement of the boxed area in *c*. (Bar = 100 μm.)

from rat optic nerves. This conclusion is further supported by the antigenic expression and proliferation potential of oligosphere cells when they were cultured in the presence of PDGF instead of B104CM, a culture condition similar to that under which O2A^{adult} cells were characterized (9, 27). It should be noted that the population expansion does not parallel the cell cycle time of oligosphere cells. This is mainly due to cell death after mechanical disaggregation and death within spheres. The slower migration in the presence of PDGF alone is potentially accounted for by the techniques used and the growth factors present. Small *et al.* (27) measured the distance a cell moved (in all directions) directly by time-lapse cinematography. We could only measured the linear distance away from the sphere. In the presence of B104CM, the adult oligosphere cells migrated in a similar velocity as neonatal oligosphere cells (13). This result suggests that adult oligosphere cells are similarly motile to neonatal oligosphere cells and that factors other than PDGF also contribute to the migration of OPs. This is further supported by the similar extent of myelination by transplanted adult oligosphere cells as by neonatal oligosphere cells (13) or by the CG4 oligodendroglial progenitor cell line (18).

Oligosphere Cells Are Derived from Neural Precursor Cells. The O2A^{adult} are derived from their neonatal counterparts (29, 31) and may regain the neonatal phenotype temporarily under certain circumstances, such as in the presence of both bFGF and PDGF (30, 32). Is the generation of neonatal-type OPs in the present study attributable to B104CM converting the adult OPs into neonatal progenitors? Our finding does not support this possibility, because the source cells (neurospheres) are nestin+ and the replacement of B104CM with PDGF in oligosphere cell cultures does not lead to the expression of the O2A^{adult} phenotype. We have attempted to generate oligospheres directly from (mechanically and enzymetically) dissociated adult (5-month-old) rat brain and optic nerves by using B104CM. The resultant culture contained floating cells that survived for up to 2 weeks in suspension but did not proliferate (data not shown). A recent observation also indicated that B104CM did not enhance the proliferation of purified O2A^{adult} progenitors (31) or convert the O2A^{adult} to O2A^{perinatal} (B. A. Barres, personal communication). B104CM is a potent mixture in selecting and propagating O2A^{perinatal} in culture (23–25). It may be speculated that some O2A^{perinatal} are selectively expanded by B104CM in the present study. The presence of O2A^{perinatal} in the adult CNS was reported based on their bipolar morphology and A2B5 positivity in a mixed culture (33). However, when the mixed glial cultures were irradiated, no O2A^{perinatal} developed (34), implying that in that study, O2A^{perinatal} cells were being generated *de novo* from A2B5-negative preprogenitor cells that were also present in the cultures. In a purified culture system, the O2A cells from adult (2-month-old) rat optic nerve displayed bipolar morphology and were immunoreactive to A2B5 (31), similar to those reported by Ffrench-Constant and Raff (33). Yet they had a very slow turnover rate (cell cycle time around 3 days), characteristic of O2A^{adult} cells. In our preparation of neurosphere cultures, these rare O2A^{perinatal} (if they are present) would be unlikely to survive in the condition without substrate and survival factors such as PDGF for a long time (>4 weeks). Our previous study (13) indicated that EGF is not a survival factor for OPs in suspension cultures. Our failure to generate oligospheres directly from dissociated adult brain and optic nerves suggests that either there are no O2A^{perinatal} present or such cells do not survive the procedure and culture condition. Therefore, the cells used for generating OPs are unlikely to contain cells that are already in the oligodendroglial lineage. Thus, the present study extends our previous argument that factors in B104CM may induce neural precursor cells to commit to oligodendroglial lineage while at the same time maintain the OPs in a state of self-renewal (13).

Multipotent Neural Precursor Cells as a Source for Remyelination. The generation and extensive propagation of the neonatal type of OPs from the adult rat brain has an important impact on the design of strategies for promoting remyelination *in vivo*. In the first instance, as we show here, it may be possible to similarly derive progenitor cells of the neonatal phenotype from the adult human brain for transplantation. Extensive animal studies suggest that transplantation of myelinating cells, especially their progenitor cells, may be an effective

approach (1, 2, 36, 37). In clinical human trials, however, cell availability becomes a problem if the cells are to be obtained from a source other than the patient. At present, human fetal tissues are the only source of immature neural cells. However, there are long-term practical and ethical concerns on the availability of such tissue, including stringent safety concerns. Here we show that it is possible to generate a large number of OPs from a small source of tissue in the rodent brain. A similar approach may be possible by biopsy from the human brain with *ex vivo* conversion of neural precursors to OPs with subsequent expansion. Such transplantation would therefore be autologous and obviate the need for immunosuppression.

The alternative approach is to recruit endogenous OPs to instigate repair. Cells that are responsible for remyelination in adults are mainly dividing "progenitor cells" (38, 39). The O4+ multiprocess-bearing cells that are regarded as the $O2A^{adult}$ *in vivo* have been found in the CNS of normal and (myelin) diseased animals and humans (4, 5, 40). The apparent lack or limit of remyelination in terms of the universal existence of O4+ $O2A^{adult}$ suggests that either the environment or the cells' intrinsic properties (or both) is responsible. In the presence of (lysolecithin-indueced) demyelination, retrovirus-labeled proliferating progenitors failed to migrate even a short distance (<500 μm) over a period of 4 weeks to perform remyelination (38). Such a poor migration behavior may be intrinsic to the multiprocess-bearing $O2A^{adult}$ rather than due to the nonpermissive environment, because transplanted neonatal OPs migrate a long distance and myelinate axons in dysmyelinated adult CNS (1, 36). Neuronal progenitors can also migrate a long distance from subependymal area to olfactory bulb in adult environment (41). In a separate study by Keirstead *et al.* (39), $O2A^{adult}$ (identified by NG2 labeling) adjacent to focally demyelinated lesions decreased in number with time and were not mitotic, and they suggested therefore that the $O2A^{adult}$ are inherently incapable of regeneration (39). Therefore, strategies designed to simply increase the number of $O2A^{adult}$, such as by delivering PDGF into the CNS (35), may not be effective. An alternative avenue to this strategy, therefore, is to promote the *in vivo* regeneration of the $O2A^{perinatal}$ from host neural precursors or stem cells, in a similar fashion as suggested by the present study. Such cells are present in subependymal areas of the adult CNS and can differentiate into neurons and glia (11), therefore close to commonly affected areas in multiple sclerosis (6). The motility of $O2A^{perinatal}$ might also indicate their ability to migrate to other parenchymal sites. The key to the application of these strategies in humans will be the identification of growth factors that have the biological effects both *in vitro* and *in vivo* on these precursor cells.

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Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats

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Limited experimental access to the central nervous system (CNS) is a key problem in the study of human neural development, disease, and regeneration. We have addressed this problem by generating neural chimeras composed of human and rodent cells. Fetal human brain cells implanted into the cerebral ventricles of embryonic rats incorporate individually into all major compartments of the brain, generating widespread CNS chimerism. The human cells differentiate into neurons, astrocytes, and oligodendrocytes, which populate the host fore-, mid-, and hindbrain. These chimeras provide a unique model to study human neural cell migration and differentiation in a functional nervous system.

Keywords: stem cell, neural progenitor cell, cell therapy

Detailed knowledge of the molecular signals controlling human precursor cell migration and differentiation is a prerequisite for the understanding of human central nervous system (CNS) development. While individual aspects of cell migration and differentiation are accessible in vitro, the molecular interactions governing these events in a complex system such as the developing CNS can be studied only in vivo. Data on neural migration and differentiation in an intact nervous system are particularly important for the design of cell replacement strategies for the treatment of human CNS disorders. An experimental model that permits the analysis of normal and disease-derived human neurons and glia in an unperturbed nervous system would greatly facilitate the study of human CNS development, disease, and repair.

Self-renewing multipotential neural stem cells can be isolated from both the embryonic and adult rodent brain and generate all three major cell types of the CNS[1–5]. Similarly, human neural precursors can be cultured in the presence of basic fibroblast growth factor (FGF2) and, upon growth factor withdrawal, differentiate into neurons, astrocytes, and oligodendrocytes[6–8]. To analyze the properties of human neural precursors in vivo, we have developed a transplant paradigm in which human cells are individually incorporated into a xenogeneic host brain without eliciting traumatic or immunological reactions. Human donor cells were not implanted into the brain tissue but merely deposited in the cerebral ventricles of embryonic rats, allowing them free access to large areas of the neuroepithelium[9–11]. The human donor cells left the ventricle and migrated in large numbers into the rat brain where they differentiated along with the endogenous cells into neurons and glia. We propose that this new approach can be used for the in vivo study of the biological properties of primary and disease-derived human neural precursors as a prelude to the design of therapeutic strategies for neurodegenerative diseases.

Results

Widespread incorporation of transplanted human precursors. Human neural precursors isolated from fetal brain fragments recovered 53–74 days postconception were transplanted immediately or after culture in defined medium containing FGF2 and/or epidermal growth factor (EGF), which promote growth of multipotent rodent neural precursors in vitro[1,2]. Cells were either grown as monolayer cultures or propagated in uncoated tissue culture dishes to form floating spheres[3,10]. In both types of cultures, differentiation into neurons, astrocytes, and oligodendrocytes could be readily induced by growth factor withdrawal[1].

Using intrauterine surgery, human donor cells were grafted into the telencephalic vesicle of embryonic day (E)17–E18 rats[11]. The transplanted cells were traced by DNA in situ hybridization with a human-specific probe to the *alu* repeat element[12] and immunohistochemistry with a human-specific antibody to glutathione-S-transferase (GSTπ). One to eight weeks after transplantation, recipients of acutely dissociated (n=12) and growth factor-treated preparations (n=32) showed incorporated human cells in a variety of gray matter regions, including olfactory bulb, cortex (Fig. 1A), hippocampus, striatum (Fig. 1B and 2), septum (Fig. 3C), tectum (Figs. 1C and 3F), thalamus (Fig. 3D), hypothalamus (Figs. 4D–F), and brain stem. Human cells were symmetrically distributed in recipient brains grafted with single cell suspensions (Fig. 3C). Animals examined during the first postnatal week also exhibited small clusters of residual donor cells attached to the ventricle walls (Fig. 3A). Transplanted spheres were entrapped in periventricular locations and gave rise to a halo of cells that migrated long distances into the host brain (Fig. 3D). Seven to eight weeks after transplantation, sphere-derived cells were found distributed over large areas of the recipient brain (Fig. 1D–E, 3F, 4A–H, 5b). Both freshly dissociated and cultured human neural precursor cells were incorporated into the host white matter. Recipient animals killed between 1 and 7 weeks of age showed abundant GSTπ-positive cells in the major fiber tracts such as the internal capsule (Fig. 1D), corpus callosum (Fig. 1D, inset), anterior and posterior commissures, stria medullaris, fornix, fimbria, as well as fiber tracts in pons and brain stem (Fig. 4G–H). In addition, several recip-

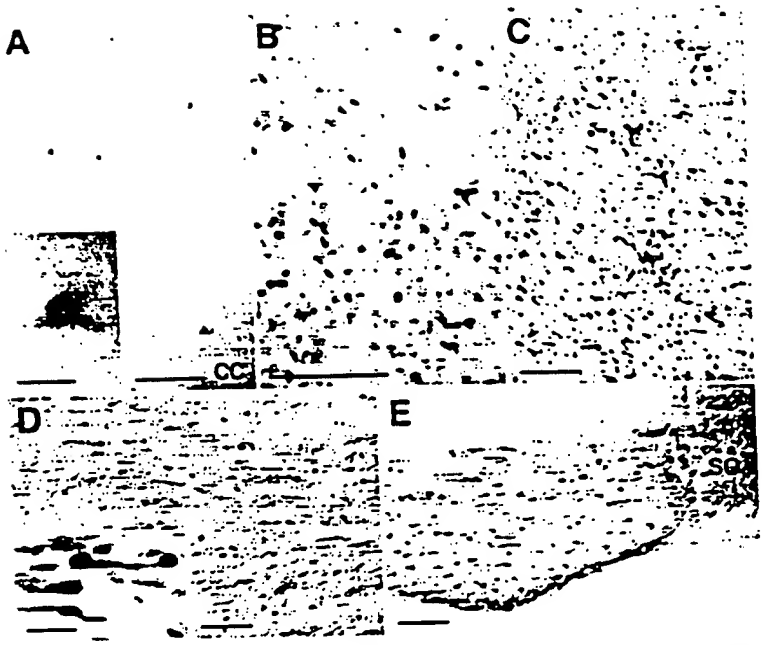

Figure 1. Incorporation of human neural precursor cells into the developing rat brain, visualized by human-specific DNA in situ hybridization (dark nuclear labeling). (A–C) Freshly dissociated cells and (D, E) cells derived from 7-week-old EGF-generated spheres. (A) cortex (postnatal day [P] 30; inset: hybridized nucleus); (B) striatum (P16); (C) inferior colliculus (P16); (D) internal capsule (P45) and corpus callosum (inset); (E) optic nerve (P45). (B), (C), and the inset in (D) are counterstained with hematoxylin to visualize host nuclei. cc: corpus callosum; so: supraoptic nucleus. Bars = 100 μm (insets: 20 μm).

ients exhibited prominent accumulations of human cells in the optic nerve (Fig. 1E). In some animals, the transplanted cells replaced large parts of the subventricular zone (SVZ) of the lateral ventricles. Two months after transplantation, these cells appeared to have migrated from the SVZ into the corpus callosum and adjacent cortical and striatal regions (Fig. 2). Numerous donor-derived cells were also found in white matter and cortex of the cerebellum (Fig. 3E). In some instances, the transplanted cells accumulated around host blood vessels or formed long chain-like structures extending into host gray and white matter (data not shown). Thus, human cells, like rodent cells[7,10,11], can engraft at various levels of the neuraxis following transplantation into the ventricle of embryonic hosts.

In vivo differentiation of human neural precursors. During the first 2 postnatal weeks, human donor cells detected with the GSTπ antibody frequently exhibited uni- or bipolar morphologies characteristic of a migratory phenotype with a leading process and a trailing cell body (Fig. 3B). Human cells incorporated into the molecular layer of the cerebellum maintained immature phenotypes with radially oriented processes for more than 7 weeks (Fig. 3E). At this stage, many of the GSTπ-labeled human cells in other brain regions had acquired multipolar oligodendroglial morphologies (Fig. 3F) and displayed immunoreactivity to an antibody recognizing oligodendrocyte-specific glycolipids (Fig. 4A–C). In addition, these cells expressed myelin basic protein (MBP) in both the cell body and within processes extending to myelin internodes, suggesting active myelination" (Figs. 4D–F). These data are compatible with studies showing

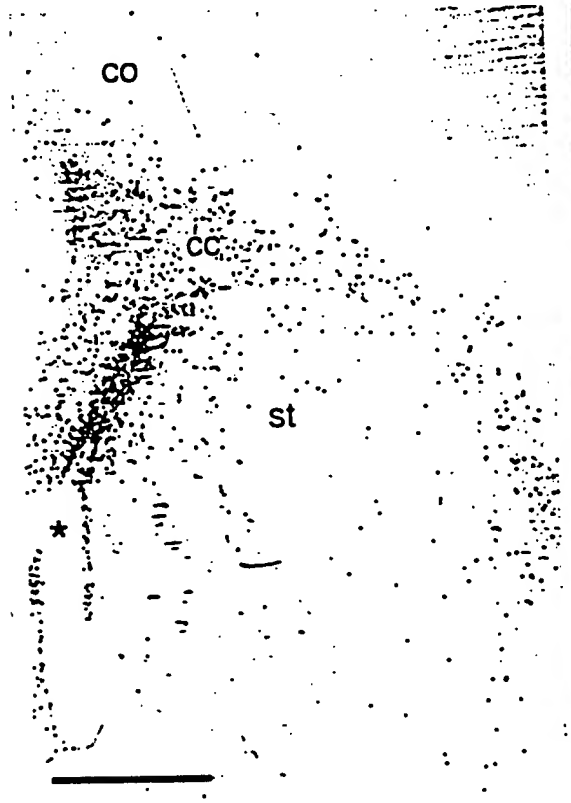

Figure 2. Human neural precursors grown for 6 weeks as monolayers in EGF- and FGF-containing media incorporated into the subventricular zone of the lateral ventricle and migrating into corpus callosum (cc), striatum (st), and cortex (co). Shown is a 50 μm vibratome section through a 7-week-old rat brain. Cells hybridized with the human *alu* probe are labeled with red dots. *lateral ventricle. Bar = 1 mm.

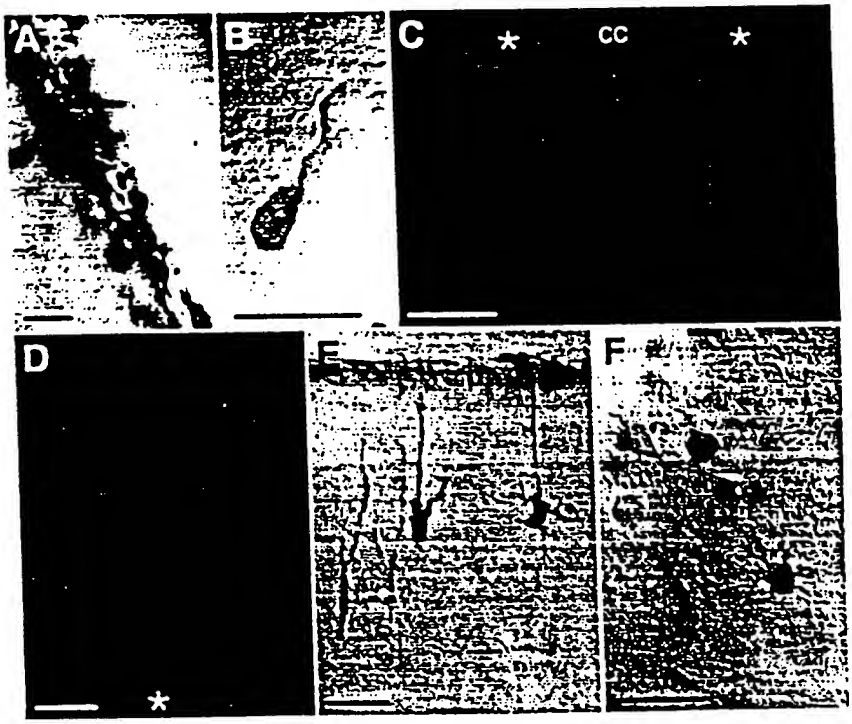

Figure 3. Morphological features of human neural precursors transplanted into the embryonic rat brain, visualized with a human-specific antibody to GSTπ. (A) Residual donor cells attached to the ventricle wall of a 3-day-old recipient animal. (B) Donor cell migrating through the striatal subventricular zone of a neonatal host. (C) Incorporation of freshly dissociated human donor cells in the septum of a 2-week-old host. (D) A human neural sphere grown for 6 weeks in EGF- and FGF-containing media incorporated into the thalamus of a 7-week-old host. (E) GSTπ-positive cells with immature radial phenotypes in the cerebellar molecular layer of a P45 animal. (F) Donor cells with multipolar oligodendroglial morphologies in the tectum, 8 weeks after transplantation of EGF-generated spheres. *ventricles; cc: corpus callosum; p: pial surface. (A, B, E, and F) immunoperoxidase; (C–D) immunofluorescence. Bars = 20 μm (A, B, E, and F), 1 mm (C), 200 μm (D).

strong GSTπ expression in rodent oligodendrocytes[7]. The presence of human oligodendrocytes was confirmed by immunohistochemical detection of the myelin protein 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) in cells hybridized with the *alu* probe (Figs. 4G and H). Some of the donor cells appeared to form CNP-positive sheaths around host axons (Fig. 4H). Although there are presently no antibodies that would distinguish human from rat CNS myelin, these patterns of MBP and CNP expression suggest that the transplanted human oligodendrocytes myelinate host axons.

Human astrocytes incorporated into the rat brain were identified by double labeling of hybridized cells with an antibody to glial fibrillary acidic protein (GFAP; Fig. 5A and B). These cells were also immunoreactive to an antibody to human adrenoleukodystrophy protein (ALDP), a peroxisomal protein strongly expressed in

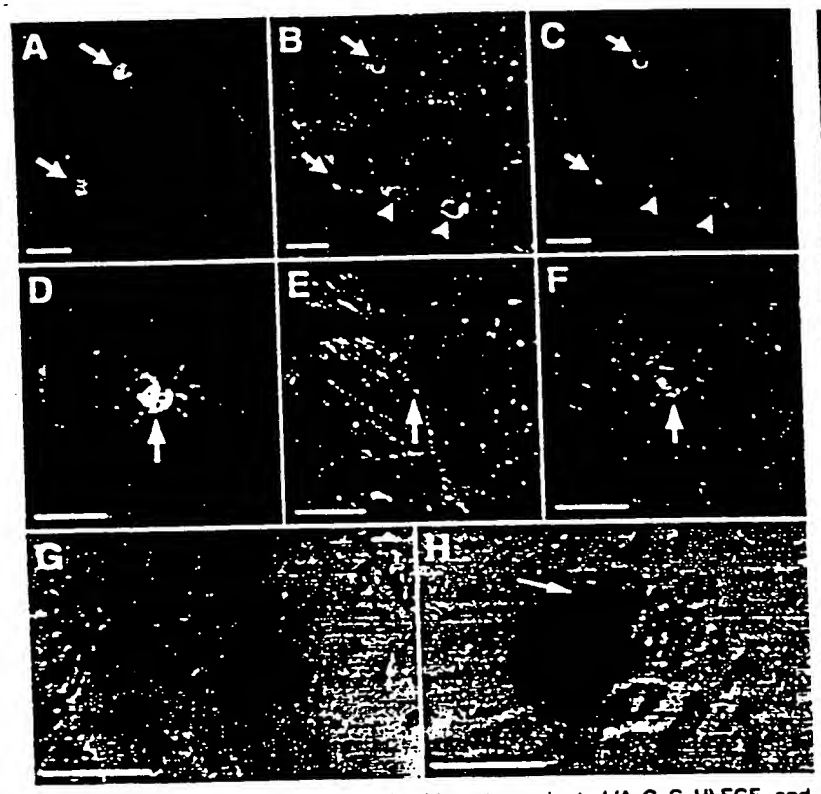

Figure 4. Human oligodendrocytes derived from transplanted (A–C, G–H) EGF- and (D-F) EGF/FGF2-generated spheres incorporated into the brain of 7-week-old rats. (A–C) Donor (arrows) and host (arrowheads) oligodendrocytes in the cortex, double labeled with antibodies to GSTπ (A and C: green) and O4 (B and C: red). (D–F) Human oligodendrocyte incorporated in the host hypothalamus, coexpressing GSTπ (D and F: green) and MBP (E and F: red). (G–H) Human oligodendrocytes in fiber tracts of the ventral brain stem, hybridized with the human *alu* probe (black) and double labeled with an antibody to CNP, which is also staining several myelin internodes (G, brown). Arrow in (H) indicates CNP staining around putative adjacent axons. (A–F) immunofluorescence confocal laser microscopy; (G–H) immunoperoxidase. Bars = 20 μm (A–F), 10 μm (G and H).

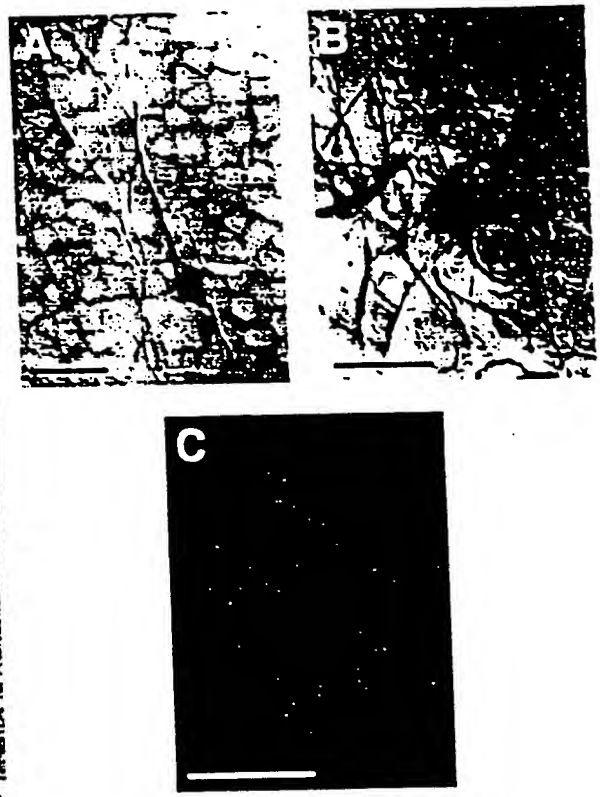

Figure 5. Astrocytic differentiation of the transplanted cells. (A) Hybridized human cell exhibiting radial GFAP-positive processes in the tectum of a 3-day-old recipient. (B) Human astrocyte with a stellate morphology in the tectum of a 7-week-old host, double labeled by in situ hybridization and an antibody to GFAP. Cells are derived from (A) FGF2- and (B) EGF-generated spheres. (C) ALDP expression in a human astrocyte in the ventral telencephalon, 18 days after transplantation of an FGF2-expanded monolayer culture into the ventricle of an E18 rat. Bars = 20 μm.

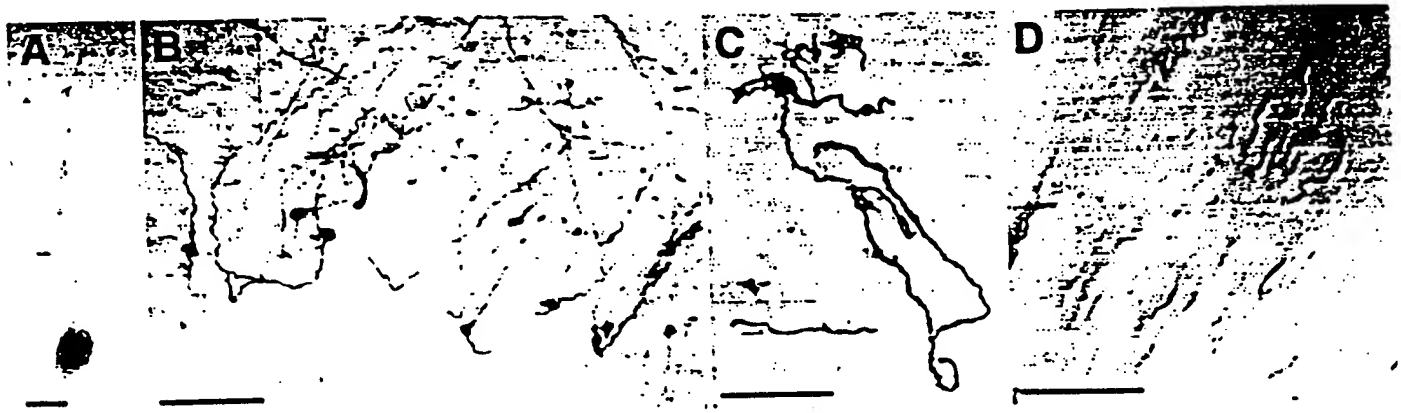

Figure 6. Human neurons incorporated into the rat brain. (A) An individual neuron, hybridized with the *alu* probe (black) and double labeled with a human-specific antibody to neurofilament (hNF-M; brown) in the cortex of a 30-day-old host grafted with freshly dissociated human precursor cells at E17. (B–C) Immunohistochemical detection of β-galactosidase-positive human cells in (B) tectum and (C) hypothalamus of 2-week-old recipients. Donor cells grown for 4 weeks in defined medium containing 10 ng/ml FGF2 were transduced with an adenovirus carrying the *lacZ* gene and transplanted into E17 recipients. (D) hNF-M-positive human axons at the transition of corpus callosum and cortex, 51 days after intraventricular transplantation of 7-week-old EGF-generated neural spheres. Bars = 10 μm (A), 100 μm (B), 50 μm (C), and 20 μm (D).

human astrocytes and microglia (Fig. 5C). Astrocytes with stellate morphologies were found in gray and white matter of forebrain, midbrain, and cerebellum. In neonatal animals, some of the donor-derived astrocytes exhibited conspicuous radially oriented processes (Fig. 5A).

A human-specific antibody to medium-sized neurofilament (hNF-M)[37] was used to visualize transplanted neurons (Fig. 6). Seven to eight weeks after transplantation, numerous immunopositive axonal profiles were detected in the host gray and white matter (Fig. 6D). Donor-derived axons were particularly abundant in cortex and within large fiber tracts such as the corpus callosum and the anterior commissure. As the human hNF-M antibody labeled mostly axons, it only occasionally allowed the identification of neuronal cell bodies (Fig. 6A). In contrast, both neuronal cell bodies and processes could be identified after transplantation of cultured cells infected with an adenovirus harboring the *lacZ* gene, an experiment done to explore the feasibility of gene transfer into transplanted human cells. Immunohistochemical detection of β-galactosidase showed neurons with polar morphologies and long axons incorporated into the host tissue (Figs. 6B and C). β-galactosidase expression was also found in cells with glial morphologies in both gray and white matter, although double immunofluorescent analyses will be required to identify the different types of human cells expressing the transgene.

Discussion

Human neural precursor cells implanted into the cerebral ventricle of embryonic rats incorporate efficiently into the host brain, generating widespread CNS chimerism. Donor cells transplanted as single cell suspensions or spheres migrate into a variety of telencephalic, diencephalic, and mesencephalic regions and differentiate into oligodendrocytes, astrocytes, and neurons. As in mouse-rat neural chimeras[34], no signs of rejection were observed up until at least 2 months after transplantation, indicating immunological tolerance of the transplanted xenogeneic cells by the embryonic rat brain. The ability to incorporate into a xenogeneic recipient brain is maintained after prolonged proliferation of the donor cells in defined, growth factor–containing medium. Moreover, donor cells transduced in vitro with an adenoviral vector continue to express the transgene after incorporation into the host brain.

Previous studies have shown that human neural precursors transplanted directly into the brain tissue of adult immunosuppressed rodents form cell clusters with limited spread of the transplanted cells into the adjacent host brain[17,18,21]. In contrast, intraventricular transplantation into embryonic hosts permits widespread delivery of human cells to many brain regions. The widespread distribution of the transplanted human cells is similar to the incorporation pattern observed after intrauterine transplantation of rodent cells[34,35,38]. In both cases, the transplanted cells appear to follow endogenous migratory routes. For example, human precursors leaving the ventricle migrated into the optic nerve, where they acquired oligodendroglial morphologies. This observation is reminiscent of the migration of oligodendrocyte precursors from the third ventricle into the optic nerve[39]. Donor cells in the SVZ of the lateral ventricle migrated into the corpus callosum and adjacent cortical and striatal regions (Fig. 2), similar to the migration of glial cells generated postnatally in the rat subventricular zone[40]. These similarities suggest that donor cell migration is not primarily determined by cell-autonomous properties but by guidance cues within the host brain. Responsiveness of human donor cells to migration cues within a rodent brain implies remarkable conservation of these signals across species.

The SVZ serves as an endogenous source of multipotential neural precursors that give rise to neurons and glia throughout life[41]. The ability to introduce human neural precursors into the rat SVZ offers an interesting perspective for the study of cell replacement in the nervous system. Future cell replacement strategies may no longer depend on cell transplantation but focus on the external modulation of endogenous neuro- and gliogenesis by gene transfer and growth factor delivery[42,43]. Incorporation of human cells into a rodent SVZ provides a unique opportunity to explore the efficacy of these strategies in vivo.

Our data suggest that many of the human cells leaving the SVZ populate the white matter and acquire oligodendroglial phenotypes (Fig. 2). Following migration, differentiated human oligodendrocytes express the myelin proteins MBP and CNP and form CNP-positive sheaths around host axons, suggesting myelin formation (Fig. 4H). Transplants into myelin-deficient mutants will determine the exact amount and distribution of human myelin within the host brain[44,45]. Thus, the model described might be used and adapted in various ways to study the mechanisms of myelin repair in human demyelinating diseases[46]. As these diseases affect large areas of the CNS, repair of myelin by transplantation would require widespread delivery of oligodendrocytes to the host brain. So far, oligodendrocyte transplants have been generally performed as intraparenchymal grafts, resulting in successful yet spatially restricted remyelination in a variety of animal models[47–49]. The strategy presented here can be exploited to optimize widespread remyelination in demyelinating diseases. Initially, this approach will be particularly relevant to the question of myelin repair in human leukodystrophies occurring in the perinatal period[50].

Combined with gene transfer into the human donor cells, this chimera model will permit the study of molecular mechanisms regulating human neural migration and differentiation in a functional brain. Alternatively, human donor cells can be introduced into embryos of transgenic mice overexpressing factors known to promote precursor cell migration and differentiation. Such a strategy can be used to assay the effect of trophic factors on human neural cells in a live nervous system. These studies should be particularly useful for the design of cell replacement strategies as well as for probing the efficacy of gene transfer protocols in the human CNS. The lack of traumatic and reactive alterations in the chimeric brains could make this approach a useful tool for the in vivo analysis of neural cells obtained from patients with neurological diseases. Incorporation of affected cells into an unperturbed nervous system may provide new insights into the cellular pathogenesis of these diseases and serve as a model to assay the effects of pharmaceutical agents on human neurons and glia in vivo.

Experimental protocol

Dissociation of human donor cells. Human fetal brain specimens were obtained with consent of the mothers from the Birth Defect Research Laboratory, University of Washington, Seattle (supported by NIH/NICHD grant HD 00836, IRB number 26-0769-A). Cerebral fragments cleaned in sterile conditions and shipped overnight in hibernation buffer[51] typically yielded cell preparations with a viability of 80–95%. Eleven specimens, obtained between 53 and 74 days postconception, were used for this study. Tissue fragments were washed five times in calcium- and magnesium-free Hanks' buffered salt solution (CMF-HBSS) and mechanically triturated to single cell suspensions in the presence of 0.1% trypsin and 0.1% DNAse (Worthington, Freehold, NJ).

Cell culture. Human neural precursor cells were grown in defined medium containing DMEM/F12 (Life Technologies, Rockville, MD), glucose, glutamine, sodium bicarbonate, 25 μg/ml insulin (Intergen, New York), 100 μg/ml human apo-transferrin (Intergen), 20 nM progesterone (Sigma St. Louis, MO), 100 μM putrescine (Sigma), 30 nM sodium selenite (Sigma), penicillin/streptomycin, 10–20 ng/ml FGF2 and/or 20 ng/ml EGF (R&D Systems, Minneapolis, MN). Monolayer cultures were propagated for 1–4 weeks in tissue culture plates coated with fibronectin (Life Technologies; 1 μg/ml) or polyornithin (Sigma; 1.5 μg/ml). Cells were passaged mechanically using a cell scraper. Immediately prior to transplantation, cells were triturated in the presence of 0.1% DNAse. Neural spheres were generated by growing

dissociated cells up to 7 weeks in uncoated tissue culture plates as described[1]. Both types of cultures yielded abundant proliferating cells that expressed nestin, an intermediate filament typically found in neural precursors[2]. Upon growth factor withdrawal, monolayer cultures and plated spheres differentiated into neurons, astrocytes, and oligodendrocytes expressing the cell type-specific antigens β-III tubulin, GFAP, and O4, respectively (A.H. unpublished observations). Growth factor treatment was continued until the day before transplantation. Selected monolayer cultures growing in 10 ng/ml FGF2 were infected with an adenoviral vector carrying the *lacZ* gene under control of the cytomegalovirus immediate-early promoter. Starting 24 h before transplantation, subconfluent 10 cm plates were incubated in 5 ml medium containing 1.5×10^7 pfu of concentrated viral supernatant. Immediately prior to transplantation, cells were washed three times in CMF-HBSS and harvested by incubation in 0.05% trypsin. Following incubation in soybean trypsin inhibitor (Life Technologies), cells were briefly triturated in the presence of 0.1% DNAse.

Intrauterine transplantation. Timed pregnant Sprague-Dawley rats (E17–E18) were anesthetized with ketamine-HCl (80 mg/kg) and xylazine (10 mg/kg), and 0.25–4×10^5 cells (suspended in 1–5 µl CMF-HBSS) were injected into the telencephalic vesicle of each embryo as described[3]. Spheres were sedimented at 150 G for 3 min, washed several times in CMF-HBSS, and implanted using a glass capillary with a 200 µm orifice (20–50 spheres per recipient brain). Larger spheres were mechanically fragmented prior to transplantation. In contrast to recipient animals grafted with mouse cells[4], human-rat neural chimeras showed a high rate of mortality within the first 2 postnatal days (30–40%). Incorporated cells were found in 31 of 44 analyzed recipient brains transplanted with acutely dissociated cells (n = 12; eight positive), monolayer (n = 13; 11 positive), or sphere cultures (n = 19; 12 positive).

Immunohistochemistry. Zero to seven weeks after spontaneous birth, recipients were anesthetized and perfused with 4% paraformaldehyde in phosphate-buffered saline (neonatal animals were fixed by immersion). Serial 50 µm vibratome sections were characterized with antibodies to GFAP (1:100; ICN Biomedicals, Costa Mesa, CA, and 1:500; Sternberger Monoclonals, Baltimore, MD), human glutathione-S-transferase (1:200; Biotrin, Dublin), human ALDP (1:400), phosphorylated medium molecular weight human neurofilament (clone HO14, 1:50), CNP (1:200; Sigma), O4 (1:5; Boehringer Mannheim, Indianapolis, IN), MBP (1:200; Boehringer), and β-galactosidase (1:500; 5Prime3Prime, Boulder, CO). Antigens were visualized using appropriate fluorophore or peroxidase-coniugated secondary antibodies. Specimens were examined on Zeiss Axioplan, Axiovert, and Laser Scan microscopes.

In situ hybridization. Donor cells were identified using a digoxigenin end-labeled oligonucleotide probe to the human *alu* repeat element[5]. DNA-DNA in situ hybridization was performed as described[6]. Briefly, sections were treated with 25 µg/ml pronase in 2× SSC, 5 mM EDTA for 15 min at 37°C, dehydrated, and denatured in 70% formamide, 2× SSC for 12 min at 85°C. After dehydration in cold ethanols, sections were hybridized overnight at 37°C in 65% formamide, 2× SSC, 250 µg/ml salmon sperm DNA. Washes were 50% formamide, 2× SSC (20 min, 37°C), and 0.5×SSC (15 min, 37°C). Hybridized probe was detected using an alkaline phosphatase-coniugated antibody to digoxigenin (Boehringer).

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Human neural stem cells

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Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes

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Stable clones of neural stem cells (NSCs) have been isolated from the human fetal telencephalon. These self-renewing clones give rise to all fundamental neural lineages in vitro. Following transplantation into germinal zones of the newborn mouse brain they participate in aspects of normal development, including migration along established migratory pathways to disseminated central nervous system regions, differentiation into multiple developmentally and regionally appropriate cell types, and nondisruptive interspersion with host progenitors and their progeny. These human NSCs can be genetically engineered and are capable of expressing foreign transgenes in vivo. Supporting their gene therapy potential, secretory products from NSCs can correct a prototypical genetic metabolic defect in neurons and glia in vitro. The human NSCs can also replace specific deficient neuronal populations. Cryopreservable human NSCs may be propagated by both epigenetic and genetic means that are comparably safe and effective. By analogy to rodent NSCs, these observations may allow the development of NSC transplantation for a range of disorders.

Keywords: cell therapy, progenitor cell, gene therapy, Tay-Sachs disease, transplanation, differentiation

Neural stem cells (NSCs) are primordial, uncommitted cells postulated to give rise to the array of more specialized cells of the central nervous system (CNS)[1–4]. They are operationally defined by their ability (1) to differentiate into cells of all neural lineages (i.e., neurons—ideally of multiple subtypes, oligodendroglia, astroglia) in multiple regional and developmental contexts; (2) to self-renew (to give rise to new NSCs with similar potential); and (3) to populate developing and/or degenerating CNS regions. The demonstration of a monoclonal derivation of progeny is obligatory to the definition (i.e., a single cell must possess these attributes). With the earliest recognition that rodent neural cells with stem cell properties, propagated in culture, could be reimplanted into mammalian brain where they could reintegrate appropriately and stably express foreign genes[5–11], gene therapists and neurobiologists began to speculate how such a phenomenon might be harnessed for therapeutic advantage as well as for understanding developmental mechanisms. These, and the studies they spawned (reviewed in refs. 14–16), provided hope that the use of NSCs might circumvent some limitations of presently available graft material[12] and gene transfer vehicles[13] and make feasible a variety of therapeutic strategies.

Neural cells with stem cell properties have been isolated from the embryonic, neonatal, and adult rodent CNS and propagated in vitro by a variety of equally effective and safe means—both epigenetic (with mitogens such as epidermal growth factor [EGF][5] or basic fibroblast growth factor [bFGF][10,17] or with membrane substrates[5]) and genetic (with propagating genes[5] such as *v-myc*[8,9] or large T-antigen [*T-Ag*][7]). Maintaining NSCs in a proliferative state in culture does not subvert their ability to respond to normal developmental cues in vivo following transplantation (such as the ability to withdraw from the cell cycle, interact with host cells, and differentiate[1,4]). These extremely plastic cells migrate and differentiate in a temporally and regionally appropriate manner particularly following implantation into germinal zones. Intermingling nondisruptively with endogenous progenitors, responding similarly to local cues for their phenotypic determination, and appropriately differentiating into diverse neuronal and glial types, they participate in normal development along the rodent neuraxis. In addition, they can express foreign genes in vivo[5–11], often in widely disseminated CNS regions[7,11], and are capable of neural cell replacement[7].

The presumption has been that the biology that endows such rodent cells with their therapeutic potential is conserved in the human CNS. If true, then progress toward human applications may be accelerated. We demonstrate the potential of clones of human NSCs to perform these critical functions in vitro and in vivo in a manner analogous to their rodent counterparts.

Results and discussion

Isolation, propagation, and cloning of human NSCs. The isolation, propagation, characterization, cloning, and transplantation of NSCs from the human CNS mirrored strategies used for the murine NSC clone C17.2 (propagated following transduction of a constitutively downregulated *v-myc*[8,9]) and for growth factor-expanded murine NSC clones[5]. NSCs—even genetically propagated clones[11]—require molecules like bFGF and/or EGF in serum-free medium to divide[1,4]. Therefore, this dual responsiveness was

chosen for both screening and enriching a starting population of stable, dissociated, cultured primary human neural tissue for cells. Cells dissociated from human fetal telencephalon—particularly the ventricular zone, which has been postulated to harbor (in lower mammals) a rich NSC population[7]—were initially grown as a polyclonal population first in serum-supplemented and then in serum-free medium containing bFGF and/or EGF. Cells were transferred between media containing one or the other of the mitogens to select for dual responsiveness. Some populations were then maintained in bFGF alone for subsequent manipulation and cloning; others were used for retrovirally mediated transduction of *v-myc* and subsequent cloning.

To provide an unambiguous molecular tag for assessing the clonal relationships of the cells, as well as to facilitate identification of some cells following transplantation and to assess their capacity to express exogenous genes in vivo, some bFGF-propagated subpopulations were infected with an amphotropic replication-incompetent retroviral vector encoding *lacZ* (and *neo* for selection). Single resistant colonies were initially isolated by limiting dilution. Monoclonality of the cells in a given colony was then confirmed by demonstrating the presence of only one copy of the *lacZ/neo*-encoding retrovirus, with a unique chromosomal insertion site. In clone H1, for example, all *lacZ/neo*-positive cells, had a single, common retroviral integration site indicating that they were derived from a single infected "parent" cell (Fig. 1A).

In rodents, genes (such as *v-myc* and *T-Ag*) that interact with cell cycle regulatory proteins have been used to propagate NSCs[8-11], neural progenitors[12], and neuroblasts[13], resulting in engraftable rodent NSC clones that can be manipulated and have therapeutic potential[14]. Therefore, some of the bFGF-maintained human cell populations, enriched for NSCs, were infected with an amphotropic, replication-incompetent retroviral vector encoding *v-myc* and *neo*[15] yielding multiple colonies. All of the putative clones had only one unique retroviral insertion site, demonstrating their monoclonality (Fig. 1B). Five clones (H6, H9, D10, C2, and E11) were generated and maintained in serum-free medium containing bFGF.

Multipotency and self-renewal in vitro. In uncoated dishes and in serum-free medium supplemented with bFGF, all clones grew in culture as clusters that could be passaged weekly for at least 1 year (Fig. 2A). The cells within these clusters expressed vimentin, a neural progenitor marker[16]. By dissociating these clusters and plating them in serum-containing medium, these clones differentiated spontaneously into neurons and oligodendrocytes (Figs. 2B and C). After 5 days under these differentiating conditions, 90% of the cells in all clones became immunoreactive for the neuronal marker neurofilament (NF; Fig. 2B); 10% expressed CNPase, a marker for oligodendroglia (Fig. 2C). Mature astroglia containing glial fibrillary acidic protein (GFAP) were not initially observed, even after 1 month under these culture conditions. However, GFAP production could be induced by coculture with primary dissociated embryonic murine CNS tissue (Fig. 2D). In addition to cells expressing the variety of differentiated lineage-specific markers (establishing "multipotency"), each clone gave rise to new immature vimentin-positive cells (Fig. 2E), which could, upon subsequent passage, give rise to new cells expressing multiple differentiated neural markers as well as to new vimentin-positive passageable cells (i.e., "self-renewability"). All the clones, whether genetically modified or epigenetically maintained, were similar in vitro.

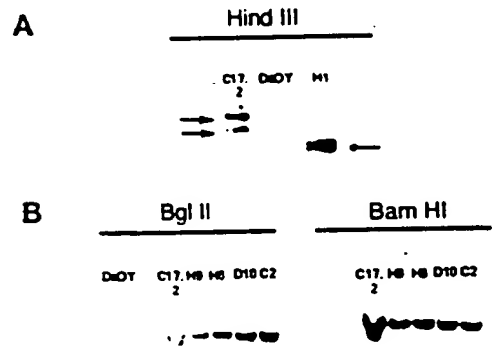

Figure 1. Southern blot analysis of retroviral insertion into human NSC clones. (A) Genomic DNA from clone H1 (propagated in bFGF and transduced with a retrovirus encoding *lacZ* and *neo*) digested with Hind III (cuts once within the provirus) and incubated with a radiolabeled *neo* probe. The murine NSC clone C17.2 contains two integrated proviruses encoding *neo*[R,R]. DaOY is an uninfected human meduloblastoma cell line. (B) Genomic DNA from clones H9, H6, D10, and C2 (propagated in bFGF and/or EGF and infected with a retrovirus encoding *v-myc*) were digested with BglII or BamHI (cuts once within the provirus) and probed for *v-myc*. C17.2 contains one *v-myc*-encoding provirus.

Ability to cross-correct a genetic defect. To assess their potential as vehicles for molecular therapies, we compared the ability of human NSCs to complement a prototypical genetic defect to murine NSCs[17]. The neurogenetic defect chosen was in the α-subunit of β-hexosaminidase, a mutation that leads to hexosaminidase-A deficiency and a failure to metabolize GM_2 ganglioside to GM_3 (Tay-Sachs disease [TSD]). Pathologic GM_2 accumulation in the brain leads to progressive neurodegeneration. The ability of human NSCs to cross-correct was compared with that of two established murine NSC clones: C17.2 and a subclone of C17.2 (C17.2H) engineered via retroviral transduction of the human α–subunit gene to overexpress hexosaminidase[18]. These murine NSC clones secrete functional hexosaminidase-A[18]. A transgenic mouse with an α-subunit deletion[19] permitted examination of the ability of human NSCs to secrete a gene product capable of rescuing TSD neural cells. NSCs (murine and human) were cocultured with dissociated TSD mouse brain cells from which they were separated by a porous membrane that allowed passage of hexosaminidase but not cells. After 10 days, the mutant neural cells were examined: (1) for the presence of hexosaminidase activity (Fig. 3A–C, and M); (2) with antibodies to the α-subunit and to CNS cell type markers to determine which TSD neural cells internalized corrective gene product (Fig. 3D–L); and (3) for reduction in GM_2 storage (Fig. 3N). While there was minimal intrinsic hexosaminidase activity in TSD cells cultured alone (Fig. 3A), hexosaminidase activity increased to normal intensity when the cells were cocultured with murine or human NSCs (Fig. 3B and C). The extent of human NSC-mediated cross-correction matched the success of murine NSCs, yielding percentages of hexosaminidase-positive TSD cells significantly greater than in untreated controls ($p<0.01$) (Fig. 3M). All neural cell types from the TSD mouse brain were corrected (Figs. 3D–L). The percentage of TSD CNS cells without abnormal GM_2 accumulation was significantly lower in those exposed to secretory products from human NSCs than in untreated TSD cultures ($p<0.01$), approaching those from wild-type mouse brain (Fig. 3N).

Multipotency and plasticity in vivo. We next determined whether human NSC clones (whether epigenetically or genetically propagated) could respond appropriately to normal developmental cues in vivo, which include migrating appropriately; integrating into host parenchyma; and differentiating into neural cell types appropriate to a given region's stage of development, even if that stage is not the one in which the NSCs were obtained. Although there are many approaches for testing these qualities[20-24], we used paradigms similar to those we have used with murine NSCs to assess their developmental ability[9]. When murine NSC clones are implanted into the cerebral ventricles of newborn mice, the cells engraft in the subventricular germinal zone (SVZ)[11] and follow the

established pathways used by endogenous progenitors, either migrating along the rostral migratory stream (RMS) to the olfactory bulb (OB), becoming neurons[7], or migrating into subcortical and cortical regions (where gliogenesis predominates and neurogenesis has ceased) becoming oligodendroglia and astroglia[8]. When transplanted into the germinal zone of the neonatal mouse cerebellum (the external germinal layer [EGL]), these same NSCs migrate inward and differentiate into granule neurons in the emerging internal granule cell layer (IGL)[9]. Following intraventricular implantation, human NSC clones emulated the developmentally appropriate behavior of their murine counterparts (Fig. 4 and 5). The engraftment, migration, and differentiation of epigenetically perpetuated clones were identical to that of *v-myc* perpetuated clones. Three of the five *v-myc* clones engrafted well (Table 1).

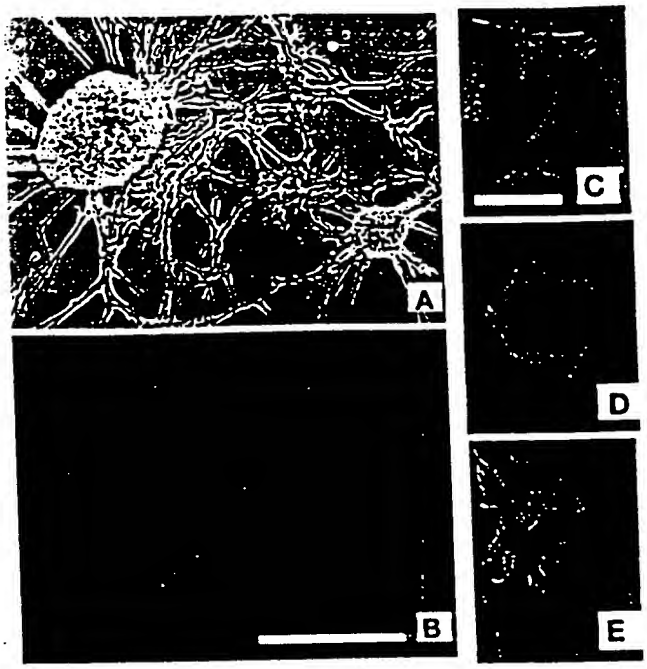

Figure 2. Characterization of human NSCs in vitro. (A) NSCs grown in serum-free medium. Immunostaining for (B) the neuronal marker neurofilament or (C) the oligodendroglia marker CNPase in serum-containing medium. (D) Immunostaining for the astrocyte marker human GFAP upon coculture with primary murine CNS cultures. (E) Immunostaining for the immature neural marker vimentin at transfer to serum-containing medium.

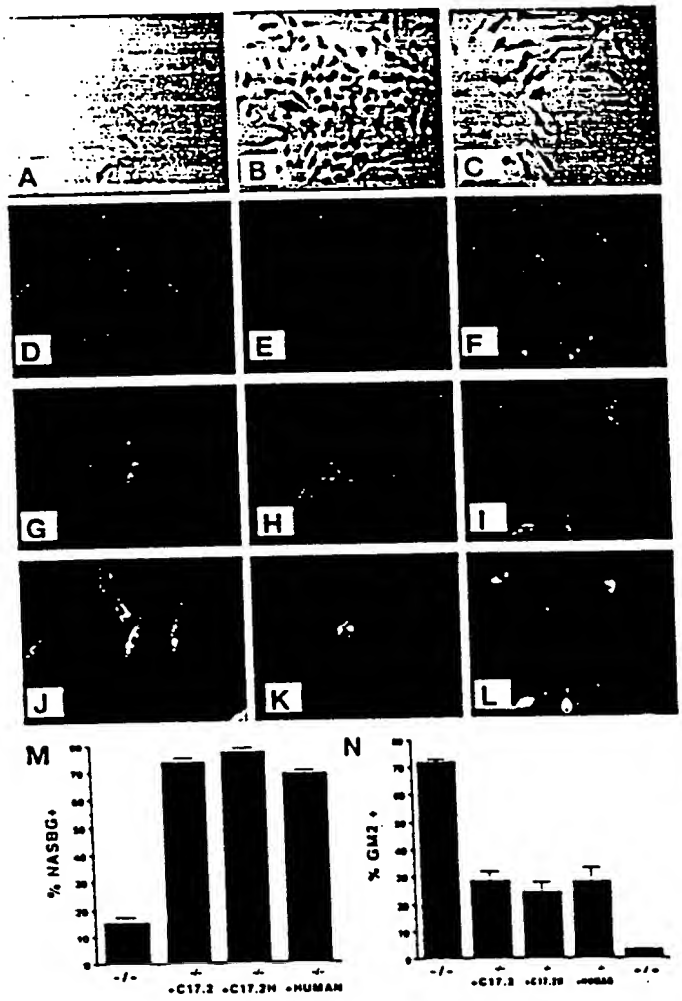

Figure 3. Dissociated brain cells from mice with mutated α-subunit of β-hexosaminidase (Tay-Sachs disease) cocultured with human NSCs. (A–C) Hexosaminidase activity determined by NASBG histochemistry. (A) TSD neural cells (arrows) not exposed to NSCs. TSD cells exposed to secretory products from (B) murine NSC clone C17.2H or from (C) human NSCs. (D–L) TSD cells cocultured with human NSCs immunostained with a (D–F) fluorescein-labeled antibody to the human α-subunit of β-hexosaminidase and (G–I) with antibodies to neural cell type-specific antigens. (G) Neuronal-specific NeuN marker; (H) glial specific GFAP marker; and (I) precursor maker, nestin. (J–L) Dual filter microscopy of the α-subunit and cell-type markers. (M) Percentage of β-hexosaminidase positive TSD cells; -/-: TSD α-subunit-null cells; TSD cells exposed to secretory products from C17.2+ murine NSCs; C17.2H+: murine NSC engineered to overexpress murine hexosaminidase; +human: human NSCs. (N) GM, accumulation in TSD cells; labels as in (M); +/+: wild-type mouse brain.

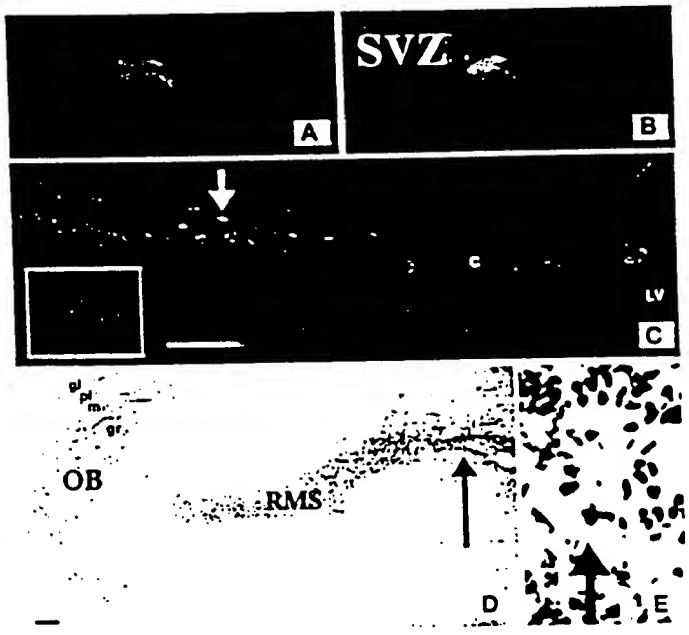

Figure 4. Migration of human NSCs following engraftment into the SVZ of newborn mice. (A,B) Human NSCs 24 h after transplantation. (A) Donor-derived cell (red) interspersed with (B) densely packed endogenous SVZ cells, visualized by DAPI (blue) in the merged image. (C) Donor-derived cells (red) within the subcortical white matter (arrow) and corpus callosum (c) and their site of implantation in the lateral ventricles (LV). Arrow indicates the cell shown at higher magnification within the inset. (D) Donor-derived cell migration from the SVZ into the rostral migratory stream (RMS) leading to the olfactory bulb (OB), in a cresyl-violet counterstained parasagittal section; gl: glomerular layer; pl: plexiform layer; m: mitral layer; gr: granular layer. Scale bars: 100 μm. (E) Higher magnification of area indicated by the arrow in (D). Brown staining indicates BrDU-immunoperoxidase-positive donor-derived cells.

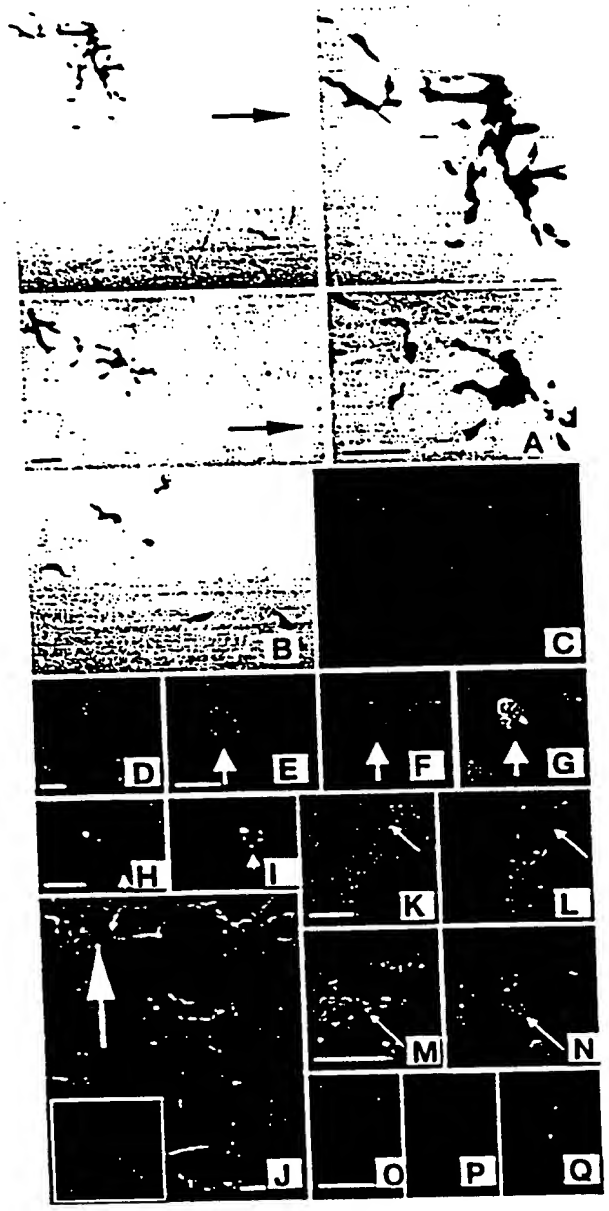

Figure 5. Characterization of human NSC clones in vivo following engraftment into SVZ of neonatal mice. (A–C) *LacZ*-expressing donor-derived cells from human NSC clone H1 detected with (A,B) Xgal and with (C) anti-β-galactosidase within (A) the periventricular and subcortical white matter regions and (B,C) OB granule layer. The arrows in (A) indicate the lateral ventricles. (D–G) BrdU-labeled NSCs (clone H6) implanted into the SVZ at birth identified in the OB with a (D) human-specific NF antibody and by (E–G) BrdU ICC via confocal microscopy. (E) BrdU-positive cell visualized by fluorescein; (F) anti-NeuN+ antibody visualized by Texas Red; (G) same cell visualized by dual filter. Donor-derived clone H6 in the adult subcortical white matter double-labeled with (H) an oligodendrocyte-specific antibody to CNPase and (I) BrdU. The arrowhead in (H) indicates a cytoplasmic process extending from the soma. (J) Donor-derived astrocytes (clone H6) in the adult subcortical white matter (indicated by the arrow) and striatum following neonatal intraventricular implantation, immunostained with a human astrocyte-specific anti-GFAP antibody. Inset is higher magnification. (K–Q) Expression of *v-myc* by human NSC clone H6 (K–N) 24 hours and (O–Q) 3 weeks following engraftment in the SVZ. (K,M,O) DAPI nuclear stains of the adjacent panels (L,N,P), immunostained for *v-myc* and (Q) immunostained for BrdU-positive donor-derived cells. (Q) is same as (P). Scale bars: (A and K): 100 μm; (D and E): 10 μm; (O): 50 μm.

Human NSCs integrated into the SVZ within 48 h following implantation (Figs. 4A and B, 5K–N). As with endogenous SVZ progenitors, engrafted human NSCs migrated out along the subcortical white matter by 2 weeks following engraftment (Fig. 4C), and, by 3–5 weeks had appropriately differentiated into oligodendrocytes and astrocytes (Fig. 5A and H–J). The ready detection of donor-derived astrocytes in vivo (Fig. 5J) contrasts with the initial absence of mature astrocytes when human NSC clones were maintained in vitro in isolation from the in vivo environment (Fig. 2D). Signals emanating from other components of the murine CNS appear necessary for promoting astrocyte differentiation and/or maturation from multipotent cells.

Endogenous SVZ progenitors also migrate anteriorly along the RMS and differentiate into OB interneurons[7]. By 1 week following transplantation, a subpopulation of donor-derived human cells from the SVZ migrated along the RMS (Fig. 4D and E). In some cases, these cells migrated together in small groups (Fig. 4E), a

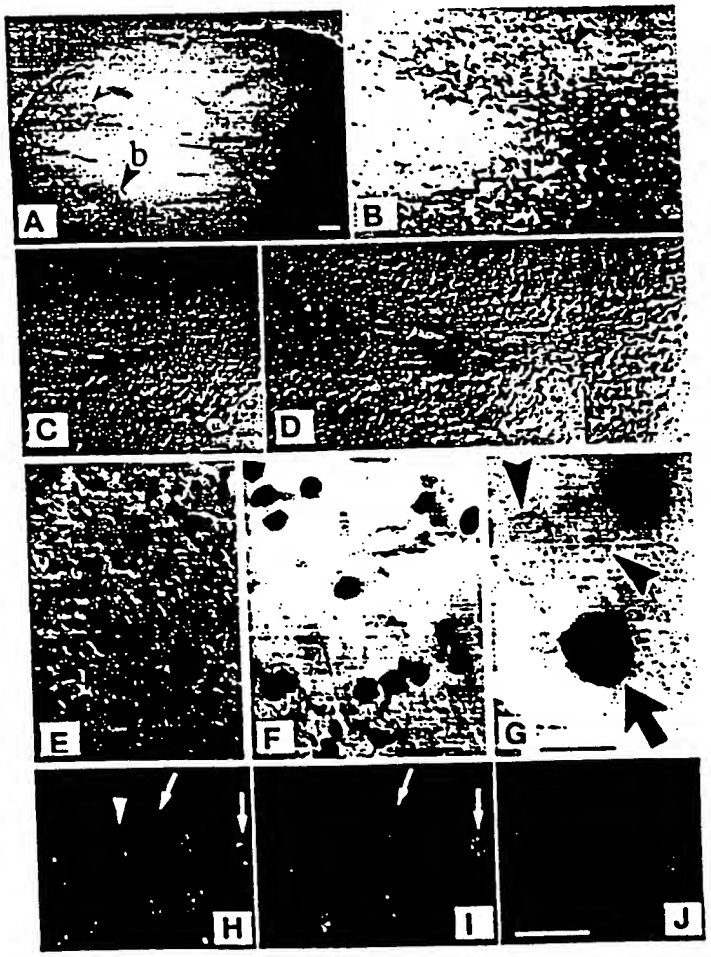

Figure 6. Transplantation of human NSCs into granule neuron-deficient cerebellum. (A–G) Donor-derived cells (clone H6) identified in the mature cerebellum by anti-BrDU immunoperoxidase cytochemistry (brown nuclei) following implantation into and migration from the neonatal *mea* EGL. (A) The internal granule cell layer (IGL and arrowheads) within the parasagittal section of the cerebellum. (B) Higher magnification of the posterior lobe indicated by "b" in (A). (C–G) Increasing magnifications of donor-derived cells within the IGL of a *mea* anterior lobe (different animal from [A,B]). (G) Normarski optics: residual host granule neurons indicated by arrowheads, representative BrDU positive donor-derived neuron indicated by the arrow. (H) Colabeling with anti-BrDU (green) and (I) NeuN (red) indicated with arrows. Arrowhead indicates BrDU+/NeuN- cell. (J) Fluorescent in situ hybridization of cells within the IGL using a human-specific probe (red). Scale bars: (A and B): 100 μm; (F, G, and J): 10 μm.

Table 1. Human neural stem cell clones.

Clone	Propagation technique	Engraftable
H1	bFGF	+
H6	v-*myc*	+
H9	v-*myc*	+
E11	v-*myc*	+
D10	v-*myc*	−
C2	v-*myc*	−

behavior typical of endogenous murine SVZ precursors[17]. Three weeks following transplantation, a subpopulation of donor-derived neurons (human-specific NF-positive cells) were present within the parenchyma of the OB, intermingled with host neurons (Fig. 5B–G). Not only were these donor-derived cells human NF-positive (Fig. 5D), but, when sections through the OB were reacted with both an antibody against BrDU (to identify prelabeled donor-derived human cells) and with an antibody to the mature neuronal marker NeuN, a large number of double-labeled BrDU+/NeuN+ donor-derived cells were integrated within the granule layer (Fig. 5E–G), mimicking the NeuN expression pattern of endogenous, host, murine interneurons (Fig. 5F and G).

Identical clones were implanted into a different germinal zone at the opposite end of the neuraxis to determine their plasiticity. Transplants of the same human NSCs into the EGLs of newborn mouse cerebella appropriately yielded different neuronal cell types in this different location, primarily cerebellar granule cells in the IGL (Fig. 6A–I), detailed below.

Therefore, in vivo—as in vitro (Fig. 2)—all engraftable human NSC clones gave rise to cells in all three fundamental neural lineages: neurons (Figs. 5D–G and 6), oligodendrocytes (Fig. 5H and I), and astrocytes (Fig. 5J). Not only did transplanted brains look histologically normal (donor cells migrated and integrated seamlessly into host parenchyma yielding no discernible graft margins), but engrafted animals exhibited no indications of neurologic dysfunction. Thus, structures that received contributions from donor human NSCs appeared to have developed normally.

Although most clones engrafted well, two appeared to engraft poorly (Table 1). Nevertheless, in vitro these clones displayed characteristics seemingly identical to those of the more robustly engrafting clones. Thus, ostensibly equivalent multipotency in vitro does not necessarily translate into equivalent potential in vivo, suggesting that each clone should be individually tested. This observation also suggests that transplantation of mixed polyclonal populations, because of their shifting representations of various clones, may be a problematic strategy.

Foreign transgene expression in vivo. Many CNS gene therapy needs require that donor cells express foreign genes in widely disseminated locations[28] (in addition to being able to do so in anatomically restricted regions[11]). Murine NSC clones have this capacity[11,12]. Human NSCs appear similarly capable. A representative retrovirally transduced, *lacZ*-expressing clone (Fig. 5A–C) continued to produce β–galactosidase after migration to, and stable integration and maturation within, host parenchyma at distant sites in the mature animal.

Spontaneous constitutive downregulation of *v-myc* expression. In the case of genetically manipulated human NSC clones, the propagating gene product *v-myc* is undetectable in donor human cells beyond 24–48 h following engraftment (Fig. 5K–Q) despite the fact that the brains of transplant recipients contain numerous stably engrafted, healthy, well-differentiated, nondisruptive, donor-derived cells (Figs. 4, 5A–J and Q, and 6). Identical findings have been observed with *v-myc*–propagated murine NSC clones[7] in which *v-myc* downregulation occurs constitutively and spontaneously and correlates with the typical quiescence of engrafted cells within 24–48 h posttransplantation. These observations suggest that *v-myc* is regulated by the normal developmental mechanisms that downregulate endogenous cellular *myc* in CNS precursors during mitotic arrest and/or differentiation. The loss of *v-myc* expression from stably engrafted NSCs following transplantation is consistent with the invariant absence of brain tumors derived from implanted *v-myc*–propagated NSCs, even after several years in mice[9,10]. As with mouse NSCs, neoplasms are never seen using human NSCs.

Neural cell replacement in vivo. Neurologic mouse mutants have provided ideal models for testing specific neural cell replacement strategies. The *meander tail* (*mea*) mutant is one such model of neurodegeneration and impaired development. *Mea* is characterized by a cell-autonomous failure of granule neurons to develop and/or survive in the cerebellum, especially in the anterior lobe[29]. Murine NSCs are capable of reconstituting the granule neuron-deficient IGL[30]. To assess whether human cells may be comparably effective in replacing neurons in CNS disorders, human NSC clones were engrafted into EGLs of newborn *mea* cerebella. When analyzed at the completion of cerebellar organogenesis, donor-derived human cells were present throughout the IGL (Fig. 6). They possessed the definitive size, morphology, and location of cerebellar granule neurons (Fig. 6E–G), identical to the few residual endogenous murine host granule neurons with which they were intermixed (Fig. 6G). That these replacement neurons were of human origin was confirmed by fluorescence in situ hybridization (FISH), using a human-specific chromosomal probe (Fig. 6J). The neuronal phenotype was confirmed by demonstrating that most engrafted cells in the *mea* IGL were immunoreactive for NeuN (Fig. 6H and I); as in the OB, endogenous interneurons in the IGL similarly express NeuN. Thus, engrafted NSCs of human origin appear sufficiently plastic to respond appropriately to varying local cues for lineage determination; recall that the donor human cells were not initially derived from a postnatal brain or from a cerebellum. Furthermore, human NSCs may be capable of appropriate neural cell replacement, much as murine NSCs are[30,31]. While many gene therapy vehicles depend on relaying new genetic information through established neural circuits—that may, in fact, have degenerated—NSCs may participate in the reconstitution of these pathways.

We have presented evidence that neural cells with stem cell features may be isolated from human brains and emulate NSCs in lower mammals[11], vouchsafing conservation of neurodevelopmental principles and suggesting that this cell type may be applied to a range of research and clinical problems in humans. NSCs may serve as adjuncts to other cellular[32], viral[33], and nonviral[34] vectors, including other human-derived neural cells[35–37]. Not only might the clones described here serve these functions, but our data suggest that investigators may readily utilize NSCs from other human material via a variety of equally safe and effective epigenetic and genetic means. That the methods used here yielded comparable cells suggests that investigators may choose the technique that best serves their needs. Insights from studies of NSCs perpetuated by one strategy may be legitimately joined to those derived from studies using others, providing a more complete picture of NSC biology and its applications.

Experimental protocol

Maintenance and propagation of human NSCs in culture. A suspension of primary dissociated neural cells (5×10^5 cells/ml), initially prepared and stably cultured from the periventricular region of the telencephalon of a 15-week human fetus[38] was plated on uncoated tissue culture dishes (Corning, Cambridge, MA) in the following growth medium: Dulbecco's Modified Eagles Medium (DMEM) + F12 medium (1:1) supplemented with N2 medium (Gibco, Grand Island, NY) to which was added bFGF (10–20 μg/ml) + heparin (5 μg/ml) and/or EGF (10–20 μg/ml). Medium was changed every 5

days. Cell aggregates were dissociated in trypsin-EDTA (0.05%) when >10 cell diameters in size and replated in growth medium at 5 x 10 cells/ml.

Differentiating culture conditions. Dissociated NSCs were plated on poly-L-lysine (PLL)-coated slides (Nunc, Naperville, IL) in DMEM + 10% fetal bovine serum (FBS) and processed weekly for immunocytochemistry (ICC). In most cases, differentiation occurred spontaneously. For astrocytic maturation, clones were cocultured with primary dissociated embryonic CD-1 mouse brain[5].

Retrovirus-mediated gene transfer. Two xenotropic, replication-incompetent retroviral vectors were used to infect human NSCs. A vector encoding *lacZ* was similar to BAG[5,6] except for the PG13 xenotropic envelope. An amphotropic vector encoding *v-myc* was generated using the ecotropic vector described for generating murine NSC clone C17.2 (ref. 20) to infect the GP + envAM12 amphotropic packaging line[7]. No helper virus was produced. Infection of bFGF- and/or EGF-maintained human neural cells with either vector (4×10^5 colony-forming units) was as described[5,6].

Cloning of human NSCs. Cells were dissociated, diluted to 1 cell/15 µl and plated at 15 µl/well of a Terasaki or 96-well dish. Wells with single cells were immediately identified. Single-cell clones were expanded and maintained in bFGF-containing growth medium. Monoclonality was confirmed by identifying a single and identical genomic insertion site by Southern blot analysis for either the *lacZ*- or the *v-myc*–encoding provirus in all progeny as described[5]. The *v-myc* probe was generated by nick translation labeling with ^{32}P dCTP; a probe to the *neo* sequence of the *lacZ*-encoding vector was generated by PCR using ^{32}P dCTP.

Cryopreservation. Trypsinized human cells were resuspended in a freezing solution comprising 10% dimethyl sulfoxide, 50% FBS, and 40% bFGF-containing growth medium and brought slowly to -140°C.

Cross-correction of mutation-induced β-hexosaminidase deficiency. The murine NSC clones C17.2 and C17.2H (ref. 22) were maintained in similar serum-free conditions as the human cells. NSCs were cocultured in a transwell system with primary dissociated neural cultures[5] from the brains of either wild-type or α-subunit null (TSD) neonatal mice[21]. These cultures were prepared under serum-free conditions, plated onto PLL-coated glass coverslips, and maintained in the medium described for NSCs. To assess production of a secretable gene product capable of rescuing the mutant phenotype, NSCs (murine and human) were cultured on one side of a membrane with 0.4 µm pores (sufficient to allow passage of hexosaminidase but not cells). The membrane was immersed in a well at the bottom of which rested the coverslip. After 10 days, coverslips were examined for hexosaminidase activity: for expression of the α-subunit in cells of various CNS lineages; for reduction in GM, storage. Hexosaminidase activity was assayed by standard histochemical techniques using the substrate napthol-AS-BI-*N*-acetyl-β-D-glucuroniside (NASBG)[23]; cells stain increasingly pink-red in direct proportion to their enzyme activity. NASBG staining of dissociated wild-type mouse brain cells served as a positive control for both intensity of normal staining and percentage of NASBG-positive cells (~100%). Neural cell types were identified by ICC with antibodies to standard markers: for neurons, NeuN (1:100; gift of R. Mullen, Chemicorp, Temecula, CA); for astrocytes, GFAP (1:500; Sigma, St. Louis, MO); for oligodendrocytes, CNPase (1:500; Sternberger Monoclonals, Baltimore, MD); and for immature undifferentiated progenitors, nestin (1:1000; Pharmingen, San Diego, CA). The α-subunit of human β-hexosaminidase was detected with a specific antibody[24]. Cells were assessed for dual immunoreactivity to that antibody and to the cell type-specific antibodies to assess which TSD CNS cell types had internalized enzyme from human NSCs. Intracytoplasmic GM, was recognized by a specific antibody[25].

Transplantation. For some models, each lateral ventricle of cryoanesthetized postnatal day 0 (P0) mice was injected as described[5] with 2 µl of NSCs suspended in phosphate buffered saline (PBS) (4×10^5 cells/µl). For other models, 2 µl of the NSC suspension were implanted into the EGL of each cerebellar hemisphere and the vermis as described[26]. All transplant recipients and untransplanted controls received daily cyclosporin 10 mg/kg given intraperitoneally (Sandoz, East Hanover, NJ) beginning on day of transplant. CD1 and *nca* mouse colonies are maintained in our lab.

Detection and characterization of donor human NSCs in vivo. Brains of transplanted mice were fixed and cryosectioned as described[5] at serial time points: P1, P2, and weekly through 5 weeks of age. Prior to transplantation, some human cells were transduced with *lacZ*. To control for and circumvent the risk of transgene downregulation, cells were also prelabeled either by in vitro exposure to BrDU (20 µM; 48 h prior to transplantation) and/or with [illegible] vital fluorescent membrane dye PKH-26 (immediately prior to transplantation as per Sigma protocol). Engrafted cells were then detected, as appropriate, by Xgal histochemistry[26]; by ICC with antibodies against β-galactosidase[27] (1:1000, XXX, Durham, NC), BrDU (1:10; Boehringer, Indianapolis, IN), human-specific NF (1:150; Boehringer), and/or human-specific GFAP (1:200; Sternberger Monoclonals); by FISH using a digoxigenin-labeled probe complementary to regions of the centromere present uniquely and specifically on all human chromosomes (Oncor, Gaithersburg, MD); and/or by PKH-26 fluorescence (through a Texas Red [TR] filter), with nondiffusibility having been verified for NSCs. Cell type identity of donor-derived cells was also established as necessary by dual staining with antibodies to neural cell type-specific markers: anti-NF (1:250; Sternberger) and anti-NeuN (1:20) to identify neurons; anti-CNPase (1:200–1:500) to identify oligodendrocytes; and anti-GFAP (1:150) to identify astrocytes. Immunostaining used standard procedures[28] and a TR-conjugated secondary antibody (1:200; Vector, Burlingame, CA). Immunoreactivity to human-specific antibodies also used standard procedures and a fluorescein-conjugated antimouse IgG secondary antibody (1:200; Vector). To reveal BrDU-intercalated cells, tissue sections were first incubated in 2N HCl (37°C for 30 min), washed twice in 0.1 M sodium borate buffer (pH 8.3), washed thrice in PBS, and permeabilized before exposure to anti-BrDU. Immunoreactivity was revealed with either a fluorescein-conjugated (1:250; Jackson, West Grove, PA) or a biotinylated (1:200; Vector) secondary antibody. *V-myc* expression (unique to donor-derived cells) was assessed with an antibody to the protein (1:1000; UBI, Lake Placid, NY). To visualize cellular nuclei, sections were incubated in the blue fluorescent nuclear label DAPI (10 min at 20°C). FISH for the human-specific centromere probe was performed on cryosections from 4% paraformaldehyde/2% glutaraldehyde-fixed brains that were permeabilized, incubated in 0.2 N HCl, exposed to proteinase K (100 µg/ml in 0.1M Tris, 0.005 M EDTA [pH 8.0]), washed (0.1% glycine), and rinsed (50% formamide/2× SSC). Probe was then added to the sections, which were coverslipped, denatured (100°C for 10 min), hybridized (15 h at 37°C), and washed (per manufacturer's protocol). Probe was detected by an antidigoxigenin TR-conjugated antibody (Boehringer) diluted 1:5 in 0.5% bovine serum albumin + 5% normal human serum in PBS. For some donor cells, multiple detection techniques were performed.

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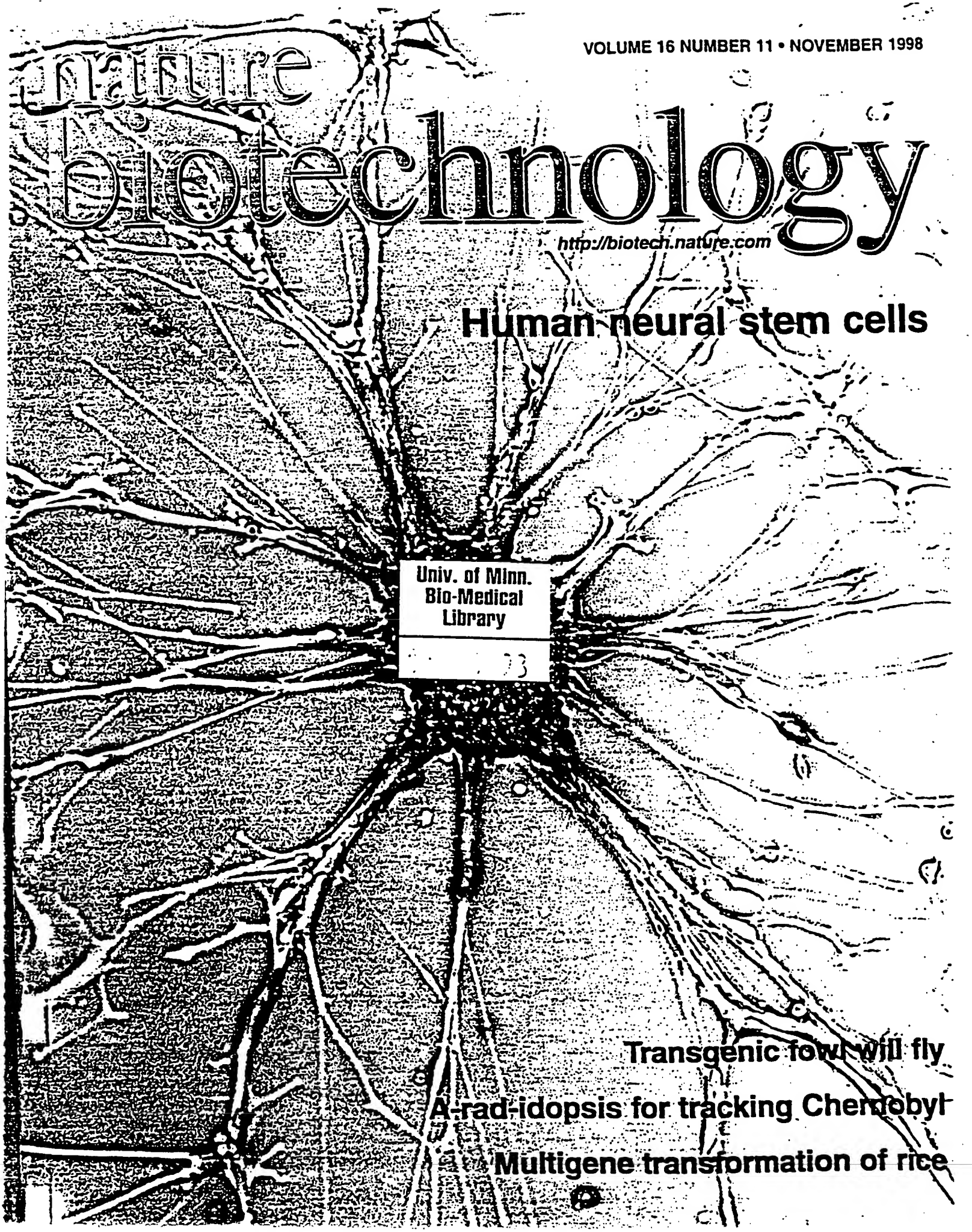

Human neural stem cells

Transgenic fowl will fly

A-rad-idopsis for tracking Chernobyl

Multigene transformation of rice

The rising star of neural stem cell research

Tanja Zigova and Paul R. Sanberg

A neuroscientist, gazing up at the night sky full of glittering stars—some of them bright and very close, others more distant and barely visible—cannot help but be struck by the similarity to brain sciences. Like stars, many aspects of neuroscience are clearly discernible, whereas others remain hidden from sight, beyond the realm of current knowledge. Ultimately, it will be necessary to piece together all this information to obtain an accurate picture of the nervous system and its inner workings. In this context, the rapidly growing field of neural progenitor and stem cell biology promises to become an increasingly bright star in the coming years. Two papers in this issue[1,2] provide a hint of the potential of neural stem cell approaches in replacement therapy and as candidates for central nervous system (CNS) gene therapy paradigms.

Over the years, enormous attention has focused on understanding the developmental origins of the nervous system. Scientists have postulated the existence of a single "stem" cell—a mother or queen of all cells—that is self-renewable and multipotent (i.e., capable of generating various committed progenitor cells and ultimately differentiating into mature cells)[3]. A neural stem cell (NSC) is defined as a single cell with the ability to proliferate, exhibit self-maintenance or renewal over the lifetime of the organism, generate a large number of clonally related progeny, retain its multilineage potential over time, and produce new cells in response to injury or disease[4]. This last criterion has proved particularly troublesome for neurobiologists.

As it is difficult to determine whether a cell within the brain can display all of the features listed above, investigators usually prefer to use terms like "putative stem cells," "stem-like cells," or "multipotent progenitors" to define mitotic cells that do not seem to be terminally differentiated and can give rise to cells of multiple neural lineages. The interest in neural stem cell biology derives, however, not only from its importance in understanding neural development, but also in its potential for providing therapies to combat neurodegenerative disease.

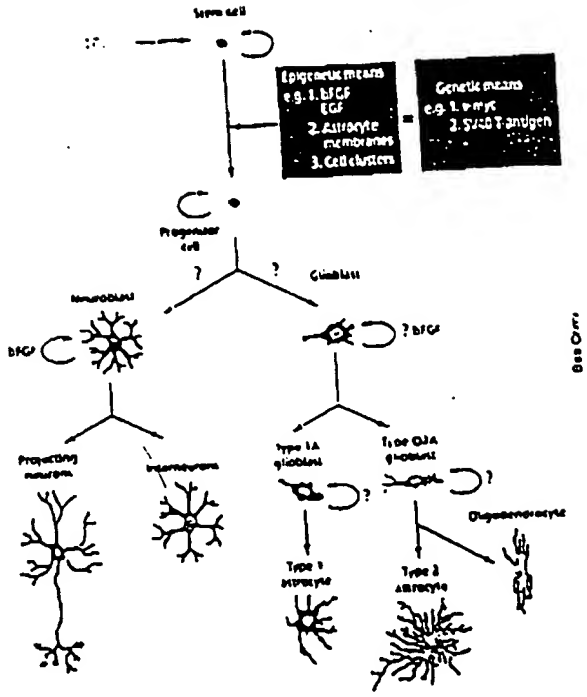

Figure 1. Lineage of neural stem cells present in fetal brain. A single stem cell capable of extended self-renewal (indicated by the arrow that loops around the top of the cell), gives rise to progenitor cells that then generate neuroblasts (neuronal precursors) or glioblasts (glia precursors). These precursors give rise to different types of neurons and glia. Factors that influence cells at particular stage of differentiation as well as epigenetic and genetic means of propagation (Snyder's study) have been indicated. Figure adapted from ref. 10.

Until now, almost all our knowledge about the properties of NSCs has been based on studies using cells originating from embryonic, neonatal, or adult rodent CNS. The report by Evan Snyder and his collaborators[1] in this issue provides strong evidence that human NSCs are able to perform in vitro and in vivo all the critical functions previously described for their rodent counterparts. The authors show that the clones of neural cells isolated from ventricular zone of the human fetal telencephalon, particularly the ventricular zone, can be safely and equally effectively propagated by either epigenetic (basic fibroblast growth factor; bFGF) or genetic (constitutively downregulated v-*myc*) means. Importantly, these clones behave identically. After plating into serum-containing medium, they differentiate spontaneously into neurons and glia, thus fulfilling the requirements of multipotency.

In addition to cells expressing the variety of differentiated lineage-specific markers, each clone also contained new immature cells, which could be passaged again. These cells could give rise to a new population of clonally related cells, some of them expressing a variety of differentiated neural markers of multiple neural lineages, others expressing the marker of immature cells (vimentin), which are suitable for another passage, thus securing the self-renewability of multipotent clones (see Fig. 1).

To assess the potential of these cells as a vehicle for molecular therapies, Snyder and his colleagues evaluated the ability of human NSCs to correct a prototypical genetic defect in an in vitro model; the defect chosen was that underlying Tay–Sachs disease, in which the pathological accumulation of GM2 gan-

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glioside in the brain leads to progressive neurodegeneration. In their experiments, the metabolic defect in primary neural cultures could be corrected by human NSCs. The efficacy observed suggests the feasibility of using human NSCs to supply a variety of therapeutic gene products to abnormal neural cells in disease.

Snyder and his colleagues subsequently implanted the human NSC clones into the lateral ventricle of newborn mice, where they integrated into the subventricular zone. From this region, they migrated extensively, either along the subcortical white matter or along the rostral migratory stream, and differentiated into cell types developmentally appropriate for the time and region of the implantation: Oligodendrocytes/astrocytes in cortical and subcortical regions and neurons in the olfactory bulb, respectively. When implanted into the cerebellum at the opposite end of the neuraxis, they yielded different neuronal cell types, mainly cerebellar granule cells.

In addition, the human NSC clones were able to "read" the developmental cues operating in different regions of the neonatal rat brain, and after completing their migration, expressed the phenotype of one of the three fundamental neural lineages. Importantly, clones engineered ex vivo by a retroviral vector to express an exogenous gene could express that gene in vivo, further establishing their efficacy for molecular gene therapy.

Snyder's group also studied the effectiveness of human NSCs to replace neurons in a neurological mouse mutant of cell deficiency (somewhat like "ablation"). In the meander tail (*mea*) mutant, characterized by a failure of granule neurons to develop and survive in cerebellum[3], engrafted clones of human NSCs were able to replace missing neurons and intermix with residual endogenous murine host granule neurons. The plasticity of human NSCs to respond to certain local cues is even more impressive as they were not harvested from the postnatal brain or cerebellum, but from the periventricular region of the fetal telencephalon, an area which presumably does not normally give rise to cerebellar granule neurons.

Important and complementing observations on the properties of human progenitor cells are outlined in an accompanying article by Ron McKay and colleagues[5]. In their study, fetal human donor cells, albeit of unknown clonal relationship, are deposited in the cerebral ventricles of embryonic rats, allowing them free access to large areas of the neuroepithelium. One to eight weeks after transplantation, recipients of acutely dissociated and bFGF/epidermal growth factor-treated preparations showed incorporated cells in a variety of grey and white matter regions, where they differentiated into all three major cell types. This incorporation pattern—previously described after intrauterine transplantation of rodent cells[6]—suggests that donor cell migration is not primarily determined by cell-autonomous properties, but rather by guidance cues within the host brain. Responsiveness of human donor cells to migration cues within a rodent brain implies remarkable conservation of these signals across species.

Of particular importance is their finding that transplanted human cells are able to replace large areas of the subventricular zone, which is known to serve as an endogeneous source of multipotential neural precursors giving rise to neurons and glia throughout life[7]. Thus, the incorporation of human neural precursors into the rat subventricular zone offers interesting insights into future cell replacement strategies.

Another important aspect of the study by McKay and colleagues is the finding that numerous human cells populating the white matter throughout the brain acquire an oligodendroglial phenotype and participate in the myelination of host axons. In the future, this model could be used and adapted in various ways to study the mechanism of myelin repair in human demyelinating diseases, whereby widespread delivery of oligodendrocytes would be desirable.

A glance at the literature reveals the frequency with which the term "stem cell" has been used rather loosely and inappropriately[8]. Snyder's study emphasizes clonality, the key for NSC definition, allowing an assessment of the true potential of individual cells. This will be an even more urgent requirement when it comes to therapeutic applications. The choice of the propagation technique, epigenetic or genetic, will depend on particular research or clinical problems. On the basis of the results of the Snyder paper, they are equally safe and effective. Yet both these approaches warrant careful scrutiny before going into humans. Knowing that both methods are giving comparable results will allow the pooling of data from various laboratories in order to apply them to human clinical neurobiology, with the hope that one day, the NSC could become the brightest of all stars in the new millennium of neuroscience and brain repair.

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Site-Specific Migration and Neuronal Differentiation of Human Neural Progenitor Cells after Transplantation in the Adult Rat Brain

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Neural progenitor cells obtained from the embryonic human forebrain were expanded up to 10^7-fold in culture in the presence of epidermal growth factor, basic fibroblast growth factor, and leukemia inhibitory growth factor. When transplanted into neurogenic regions in the adult rat brain, the subventricular zone, and hippocampus, the *in vitro* propagated cells migrated specifically along the routes normally taken by the endogenous neuronal precursors: along the rostral migratory stream to the olfactory bulb and within the subgranular zone in the dentate gyrus, and exhibited site-specific neuronal differentiation in the granular and periglomerular layers of the bulb and in the dentate granular cell layer. The cells exhibited substantial migration also within the non-neurogenic region, the striatum, in a seemingly nondirected manner up to ~1–1.5 mm from the graft core, and showed differentiation into both neuronal and glial phenotypes. Only cells with glial-like features migrated over longer distances within the mature striatum, whereas the cells expressing neuronal phenotypes remained close to the implantation site. The ability of the human neural progenitors to respond *in vivo* to guidance cues and signals that can direct their differentiation along multiple phenotypic pathways suggests that they can provide a powerful and virtually unlimited source of cells for experimental and clinical transplantation.

Key words: progenitor cells; human; transplantation; neuron; subventricular zone; dentate gyrus; striatum

The limited capacity for structural repair in the mammalian brain is in part explained by the inability of the mature CNS to generate new cellular elements in response to damage. Cell transplantation offers a possibility to circumvent this limitation. Both rodent and primate experiments show that neuroblasts and young postmitotic neurons obtained from defined parts of the neuraxis during development can survive, mature, and grow extensive functional axonal connections after transplantation to brain-damaged recipients, and both structurally and functionally replace lost neurons in the mature brain (for review, see Dunnett and Björklund, 1994). Because of the limited migratory capacity of the differentiated cells, however, these types of implants are unable to integrate into the cellular architecture of the host.

Previous studies have shown that less differentiated precursor cells, taken at premigratory stages of neuronal development, can make use of available substrates or pathways for migration, mix with endogenous pools of precursors, and participate in ongoing neurogenesis, both during development (McConnell, 1988; Gao and Hatten, 1994; Zigova et al., 1996) and in areas of the mature brain, the anterior subventricular zone (SVZa), and the hippocampal dentate gyrus, where neurogenesis continues into adulthood (Lois and Alvarez-Buylla, 1993; Vicario-Abejon et al., 1995). Similarly, mixed precursor cell populations, injected *in utero* into the developing forebrain, integrate across the ventricular wall and undergo site-specific migration and neuronal differentiation in widespread brain regions (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995), suggesting that undifferentiated progenitors may be an interesting source of cells for intracerebral transplantation.

Recently, neural progenitors with the capacity to give rise to all major cell types of the mature CNS have been isolated from the developing or adult CNS (Weiss et al., 1996b; Alvarez-Buylla, 1997; Luskin et al., 1997; Ray et al., 1997). They become more restricted in number during development and remain as a small, relatively quiescent population of dividing cells in the subventricular regions of the adult CNS. These neural progenitors can be grown *in vitro* in the presence of either epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF, FGF-2), as a population of continuously dividing progenitors capable of differentiating into both neurons and glia (Murphy et al., 1990; Reynolds and Weiss, 1992a,b, 1996; Richards et al., 1992; Ray et al., 1993; Vescovi et al., 1993; Sensenbrenner et al., 1994; Palmer et al., 1995). Cells isolated from the rat hippocampus in the presence of bFGF have been shown to express region-specific migration and neuronal differentiation after transplantation to the adult rat brain (Gage et al., 1995; Suhonen et al., 1996). Embryonic mouse or rat forebrain progenitors expanded in the presence of EGF, by contrast, develop into predominantly glial phenotypes *in vivo*, as observed after transplantation to the adult rat spinal cord (Hammang et al., 1997) or the developing rat forebrain (Winkler et al., 1998).

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Here, we have examined the question of whether progenitors isolated from the developing human CNS can exhibit *in vivo* neurogenic properties after implantation into the brain of adult recipients. Cells obtained from the forebrain of 6.5- to 9-week-old human fetuses were maintained as continuously dividing cultures in the presence of EGF, bFGF, and leukemia inhibitory growth factor (LIF). Cells expanded 10^3-10^7-fold in culture (over 9–21 passages) survived well after transplantation to both neurogenic and non-neurogenic sites; cells contained within these grafts showed migration, integration, and site-specific differentiation into both neurons and glia.

MATERIALS AND METHODS

Generation and in vitro culture of human progenitor cells. Generation of the human progenitor cell lines has been described previously (Carpenter et al., 1999). Embryonic brain tissue was obtained from one 6.5 week and one 9 week embryo (post-conception) under compliance with National Institutes of Health guidelines, Swedish government guidelines, and the local ethics committee, and appropriate consent forms were used. Tissue from the forebrain was dissected in sterile saline and transferred to N2 medium, a defined DMEM/F12-based medium (Life Technologies, Grand Island, NY) containing 0.6% glucose, 25 μg/ml human insulin, 100 μg/ml human transferrin, 20 nM progesterone, 60 μM putrescine, 30 nM selenium chloride, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM HEPES, and 2 μg/ml heparin (Sigma, St. Louis, MO). The tissue was dissociated using a standard glass homogenizer, and the dissociated cells were grown on uncoated plastic T75 flasks in N2 medium containing human EGF (hEGF, 20 ng/ml; Life Technologies), human basic FGF (hbFGF, 20 ng/ml; Life Technologies), and human LIF (hLIF) (10 ng/ml, R+D Systems, UK), at a density of ~100,000 cells/ml.

The cells grew as free-floating clusters ("neurospheres"), and were prevented from attachment by gently knocking the flasks each day. Any cells that adhered to the plastic and began to extend processes were not removed by this procedure and therefore were not carried through to the next passage. The spheres were passaged by mechanical dissociation every 7–10 d and reseeded as single cells at a density of ~100,000 cells/ml. The cells used for transplantation (the 6.5FBr and 9FBr cultures) had been expanded over 9–21 passages, which corresponds to a total increase in cell numbers of $\sim 10^3$ at 9 passages to at least 10^7 at 21 passages (Carpenter et al., 1999).

Labeling methods and preparation of cells for transplantation. To enable the detection of the cells *in vivo*, cultures were labeled with 1 μM bromodeoxyuridine (BrdU), which was added to the culture medium 48 hr before the preparation of the cells for transplantation. This resulted in ~80% labeling efficiency, with no apparent changes in growth rate of the spheres.

Cells were taken for transplantation 4–5 d after the last passage as small spheres of 5–30 cells. The spheres were collected by centrifugation at 1000 rpm for 3 min and resuspended in 1 ml DMEM/F12 medium. To check the cell viability, an aliquot of the sphere suspension was removed and mixed with trypan blue. After this was ascertained, a second cell count was performed by triturating the trypan blue aliquot to give single cells. The sphere suspension was centrifuged a second time and resuspended in a smaller volume to give the equivalent of ~100,000 cells/μl.

Transplantation. Adult female Sprague Dawley rats (B&K Universal, Stockholm, Sweden), weighing ~250 gm at the beginning of the study, were used. They were caged in groups of two and maintained on a 12 hr light/dark cycle with constant temperature and humidity, with *ad libitum* food and water. The animals were immunosuppressed throughout the experiment by daily injections of 10 mg/kg cyclosporin, beginning 1 d before transplantation.

Stereotaxic surgery was performed under deep equithesin anesthesia (3 ml/kg body weight, i.p.). Rats received 1 μl cell suspension bilaterally in either the SVZa, rostral migratory stream (RMS), or hippocampus, or 2 μl in the striatum, according to the following coordinates: SVZa, anterior (A) = +1.6, lateral (L) = ±1.5, ventral (V) = −4.2; RMS, A = +3.7, L = ±1.5, V = −5.0; hippocampus, A = −3.6, L = ±2.0, V = −3.0, −2.6; striatum, A = 0.6, L = ±2.8, V = −4.8, −4.2. The tooth bar was set at −2.3, and all ventral coordinates were taken from dura. Cells were implanted via a glass capillary (inner diameter ~70 μm) attached to a 2 μl Hamilton syringe. For the SVZa transplants, 100,000 cells from the 6.5FBr cell line were transplanted, and the brains were analyzed after 6 weeks (n = 10). For the RMS transplants, 100,000 cells were transplanted, and the brains were analyzed at either 2 weeks (6.5FBr, n = 10; 9FBr, n = 4) or 6 weeks (6.5FBr, n = 10). Both cell lines were transplanted to either the striatum (200,000 cells) or hippocampus (100,000 cells), and the brains were analyzed at either 2 or 6 weeks (n = 10 per group).

Tissue processing. At either 2 or 6 weeks after transplantation, rats were terminally anesthetized with 5% chloral hydrate and transcardially perfused with 0.1 M PBS followed by 5 min rapid fixation with ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were removed and placed in PFA overnight, before being transferred to 25% sucrose in PBS. Coronal or sagittal sections were cut on a freezing microtome at a thickness of 30 μm. In each case, eight series were collected for further processing.

Immunocytochemistry. For BrdU labeling, all sections were pretreated with 1 M HCl for 30 min at 65°C. Sections were incubated in primary antibodies for 36 hr at 4°C. All primary antibodies were diluted in 0.02 M potassium PBS (KPBS) containing 5% normal serum of the species in which the secondary antibody was raised and 0.25% Triton X-100, except for 32 kDa dopamine- and cAMP-regulated phosphoprotein (DARPP-32) and tyrosine decarboxylase (GAD_{67}) in which Triton X-100 was omitted. Antibodies used in this study were BrdU rat monoclonal (1:100, Chemicon, Temecula, CA), mouse monoclonal (1:25, Becton Dickinson, Franklin Lakes, NJ), β-tubulin-III (1:400, Sigma), calbindin (1:1000, Sigma), DARPP-32 (1:20,000; Dr. P. Greengard, Rockefeller), GAD_{67} (1:1000, Chemicon), glial fibrillary acidic protein (GFAP, 1:500, Dakopatts), RNA binding protein (Hu, 1:1000; Dr. S. Goldman, Cornell), neuronal nuclei (NeuN, 1:100, Chemicon), tyrosine hydroxylase (TH, 1:500, PelFreeze, Rogers, AR), Vimentin (VIM, 1:25, Dakopatts), and human-specific tau (hTau, 1:100, Calbiochem, La Jolla, CA). For all immunohistochemical procedures, adjacent sections served as negative controls and were processed using identical procedures, except for incubation without the primary antibody in each case.

For fluorescent double-labeling immunocytochemistry, after rinses in KPBS containing 2% of the normal serum, sections were incubated in the secondary antibodies (1:200). For rat anti-BrdU this was donkey anti-rat conjugated to FITC or Cy2 (Jackson); for mouse anti-BrdU, donkey anti-mouse conjugated to FITC or Cy2 (Jackson); for all other primary antibodies raised in mouse, rat-absorbed biotinylated horse anti-mouse (Vector); and for all primary antibodies raised in rabbit, biotinylated swine anti-rabbit (Dakopatts). All secondaries were diluted in KPBS containing 2% normal serum, and sections were reacted for 2 hr at room temperature in the dark. After three rinses in KPBS, sections were reacted with streptavidin conjugated to Cy3 (Jackson) for a further 2 hr at room temperature in the dark.

For immunohistochemistry with hTau, sections were pretreated with 3% H_2O_2 in 10% methanol to quench endogenous peroxidase activity. Incubation in the primary antibody was performed in KPBS containing 5% normal horse serum and 0.25% Triton X-100 for 36 hr at 4°C. After three rinses in KPBS, sections were incubated in the secondary antibody: rat-absorbed biotinylated horse anti-mouse (Vector) in KPBS containing 2% normal horse serum for 2 hr at room temperature. Further washing in KPBS was followed by incubation with avidin–biotin–peroxidase complex (Vectastain, Vector), for 1.5 hr at room temperature. 3,3-Diaminobenzidine (Sigma) in 0.03% H_2O_2 in KPBS was used as the chromogen.

The sections were mounted on chrome–alum-coated slides, and the fluorescent sections were coverslipped using polyvinyl alcohol–1,4-diazabicyclo[2.2.2]octane mounting medium. The hTau slides were dehydrated in ascending alcohols and coverslipped using DPX mountant.

Confocal microscopy. Colocalization of BrdU with neuronal and glial markers was conducted by confocal microscopy to enable exact definition of each of the antibodies, using a Bio-Rad MRC1024UV confocal scanning light microscope. Double-labeled cells were always verified, both by collecting optical sections of 1–2 μm throughout the specimen, and by eye, using an Olympus binocular microscope. In all figures, all double-labeled cells that are depicted were identified in this way.

RESULTS

In vitro characteristics of the transplanted cells

Two different human progenitor cell cultures obtained post mortem from the forebrain of one 6.5 week (6.5FBr) and one 9 week (9FBr) embryo were analyzed. The cells were cultured in the presence of EGF, bFGF and LIF and passaged every 7–10 d. In

these cultures bFGF was necessary to maintain continuous cell proliferation over extended time periods, and this effect was further enhanced by the addition of LIF. Parallel *in vitro* experiments (Carpenter et al., 1999) indicate that LIF promotes the sustained proliferation of the human progenitors in the neurosphere cultures. Moreover, in agreement with previous findings (Satoh and Yoshida, 1997), the proportion of cells that differentiated into neurons appeared to be increased in the presence of LIF.

The *in vitro* characteristics of the 6.5FBr and 9FBr progenitor cell cultures have been presented in detail elsewhere (Carpenter et al., 1999). Briefly, both cultures showed a growth rate that was similar to each other and to other human progenitor cell cultures derived from different gestational ages. Cells within undifferentiated spheres were immunopositive for the immature cell marker nestin and were shown to incorporate BrdU, indicative of cell division. To assess the differentiation capacity of these cells, dissociated single cells were plated onto poly-ornithine-coated glass coverslips and cultured for 12–14 d in N2 medium containing 1% FBS. On differentiation, both cell cultures demonstrated the capacity to form neurons, astrocytes, and oligodendrocytes. Immunohistochemistry using an antibody to GFAP revealed a range of 15–55% astrocytes present in both the 6.5FBr and 9FBr cultures between passage 5 (P5) and P35. An antibody to β-tubulin isotype III was used to detect neurons. At P5 the 6.5FBr cultures generated more β-tubulin-III-positive cells than the 9FBr cells (37 vs 20%, respectively). At P20–P30 (150–300 d *in vitro*), the percentage of neurons had decreased to ~15% in both cultures.

Survival and differentiation after transplantation to the adult rat brain

Cells from the 6.5FBr and 9FBr cultures were transplanted, under immunosuppression, into two neurogenic sites: the dentate gyrus of the hippocampus and the SVZa and its associated RMS, as well as to a non-neurogenic site, the striatum. Transplantation was performed using cells that had been passaged 9–21 times. The cells were labeled with BrdU during the last 48 hr before transplantation. This resulted in ~80% labeling efficiency and enabled analysis of the grafts by fluorescent immunohistochemistry using a double-labeling technique for BrdU in combination with specific neuronal and glial markers. In addition, hTau was used to identify the grafted cells.

In all animals, BrdU-positive transplanted cells were identified in all graft sites, at both 2 and 6 weeks after transplantation. Similarly, staining with the human-specific tau antibody revealed cellular and axonal profiles at all transplant sites, indicating graft survival in all cases. Extensive migration of BrdU-labeled cells, as described below, were seen in all animals where the graft deposits had been correctly placed in the RMS, SVZa, or hippocampus, respectively. No evidence of tumor formation was observed.

The transplants from both cell cultures (6.5FBr and 9FBr), regardless of the number of passages, were indistinguishable in terms of graft survival, migrational patterns, and phenotypic differentiation of the transplanted cells. Control transplants of cells that had been killed by freeze-thawing before transplantation showed no transfer of the BrdU marker to the host cells, which is in agreement with previous reports (Gage et al., 1995; Suhonen et al., 1996).

The subventricular zone and rostral migratory stream

Single deposits of 100,000 cells were deposited in or close to the SVZa, just ventral to the corpus callosum, or just above the RMS midway between the SVZa and the olfactory bulb. In the SVZa a core of BrdU-positive cells was located close to the ventricular ependyma, extending in some cases into the white matter of the overlying corpus callosum (Fig. 1*A*) (6 weeks survival). Cells were seen to leave the transplantation site in a stream of rostral migration (Fig. 1*B*) after the RMS, i.e., along the path of endogenous progenitors toward the olfactory bulb. Once they reached the bulb, BrdU-positive cells left the migratory stream, becoming dispersed throughout the subependymal, granular (Fig. 1*C*,*D*), and glomerular cell layers. The cells within the olfactory bulb were more weakly BrdU-labeled than the cells in the SVZa (which were uniformly highly labeled), suggesting that the labeled cells had undergone further cell division on their route to the bulb, similar to the endogenous progenitors from the SVZa (Menezes et al., 1995).

In the RMS transplants the deposits of BrdU-labeled cells were localized just above, and occasionally within, the RMS itself (see Fig. 4*A*). At 2 weeks after transplantation the cells remained clustered at the graft site, and there was very little migration from the graft core. Thus only few cells were observed rostral and caudal to the graft placement at this time point. Six weeks after grafting, cells were seen to have migrated rostrally toward the olfactory bulb (see Fig. 4*B*) and into the granular and periglomerular layers (see Fig. 4*C–F*).

The immature cell marker VIM was used to delineate the SVZa and RMS along which BrdU-positive cells were seen in their migratory stream (Fig. 2*A*). BrdU-positive cells were not VIM positive. The vast majority of the BrdU-labeled nuclei did not diverge from the RMS; however, in the region adjacent to the transplant core, occasional cells could be seen migrating dorsally toward the overlying cortex (data not shown). Some of the cells migrating within the RMS were double-labeled with the early neuronal markers Hu (Fig. 2*B*) and β-tubulin-III (see Fig. 4*B*). Both of these markers, which identify both early differentiated neuronal precursors and mature neurons, are known to be expressed by the endogenous progenitors from the SVZa as they migrate along the RMS (Baraban et al., 1995; Menezes et al., 1995). The presence of these markers thus indicates their early commitment to a neuronal phenotype. None of the BrdU-positive cells within the SVZa or RMS stained positively for the NeuN marker. Within the olfactory bulb, the majority of BrdU-labeled cells, both in the deeper layers and in the periglomerular layer, were Hu positive (Figs. 2*C*, 4*C*), and approximately half of the BrdU-positive cells were also double-labeled with the more mature neuronal marker NeuN (Fig. 2*D*,*E*), indicating a progressive maturation of the cells toward a neuronal phenotype as they entered the bulb. Many of the BrdU-labeled cells, within both the granule cell layer and periglomerular layer, also expressed the GABA-synthesizing enzyme GAD_{67} (see Fig. 4*D*,*E*). TH, which is a characteristic feature of the dopaminergic periglomerular neurons, was clearly expressed in some of the BrdU-labeled cells within the periglomerular layer (Fig. 4*F*, *arrowheads* and *inset*). None of the BrdU-labeled cells stained positively for the glial marker GFAP, neither within the astrocyte-rich RMS (see Fig. 2*A*) nor within the olfactory bulb (see Fig. 2*B*). In addition, no cells were double-labeled with BrdU and the receptor phosphoprotein DARPP-32, which is present in the medium spiny neurons of the striatum but not normally expressed in neurons of the olfactory bulb. Table 1 gives a semiquantitative summary of neuronal and glial differentiation of the transplanted cells within the RMS and the olfactory bulb.

Using a human-specific antibody to the cytoskeletal protein

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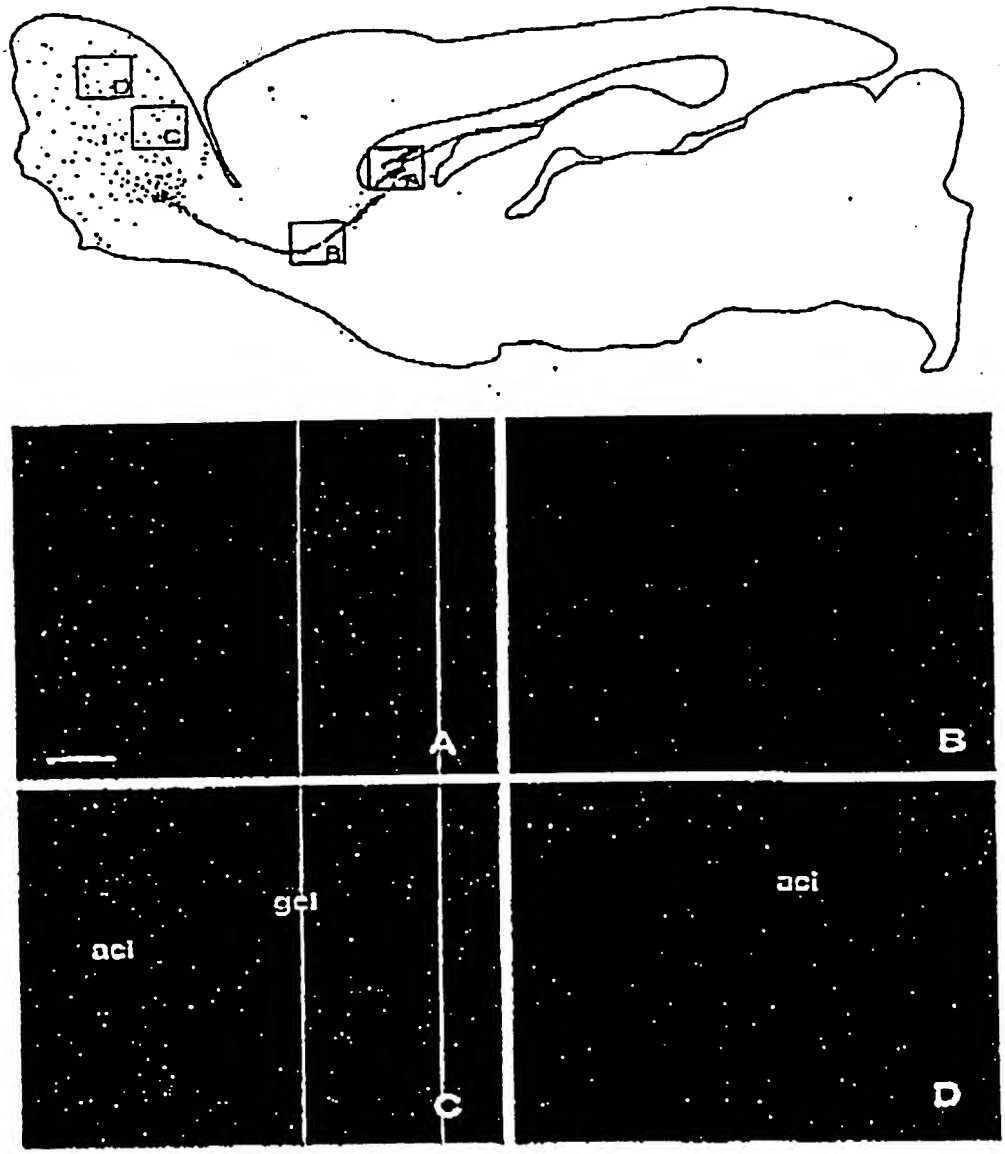

Figure 1. Low-power illustration of a transplant in the SVZa in a sagittal section, analyzed at 6 weeks after transplantation, shows an overview of the injection site of BrdU-labeled cells (*green*) and their distribution throughout the RMS. In the olfactory bulb, the cells were found dispersed through all layers. *A–D*, Grafted cells at different sites (indicated in the *top panel*), with BrdU-labeled cells shown in *green* and the NeuN shown in *red*. Double-labeled cells present in the bulb display a *yellow color* (*C*, *D*). *A*, Transplant core; *B*, cells migrating along the RMS; *C*, *D*, cells in the granule cell layer of the olfactory bulb. Scale bar, 250 μm. *aci*, Intrabulbar portion of the anterior commissure; *gcl*, granule cell layer.

tau, positive staining was observed at the injection site in both cellular and axonal profiles (Fig. 3*A*). Typically, cells that remained at the graft core or migrated only a short distance from the implantation site had developed axons that projected laterally into either the corpus callosum or striatum adjacent to the transplant (Fig. 3*A*,*B*). Tau-positive cells were distributed along the RMS, several millimeters from the graft site (Fig. 3*C*,*D*). These cells often showed a short leading process, oriented in the direction of the RMS (Fig. 4). Small tau-positive profiles were observed in the deeper layers of the olfactory bulb, and occasionally mature cells with extensive processes were found in this region (Fig. 3*E*,*F*). High background from the immunohistochemical procedure precluded the identification of tau-positive profiles in the periglomerular layer.

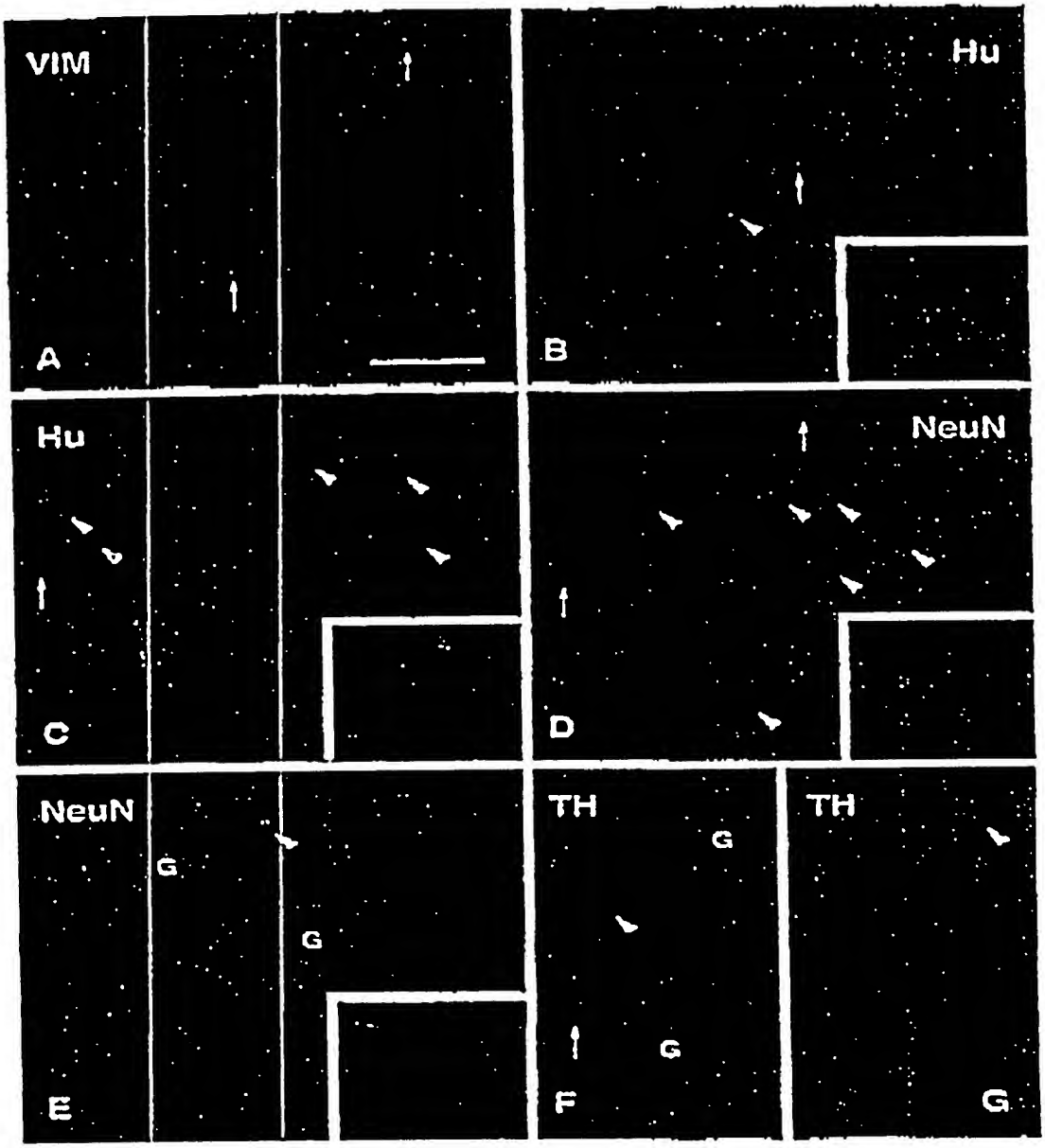

Figure 2. Confocal images of BrdU-labeled (*green*) and double-labeled cells (*yellow*) transplanted in the SVZa at 6 weeks after transplantation. *A*, Vimentin (*VIM*) staining delineated the RMS. BrdU-positive cells (*arrows*) were observed along the RMS but were not VIM positive. *B*, Many host cells present within the RMS were positively stained with an antibody to Hu, a neuronal phenotypic marker, and some transplanted cells were also Hu positive (*arrowhead*, enlarged in the *inset*). *C*, Many Hu-positive transplanted cells (*arrowheads*) were located within the granule cell layer of the olfactory bulb. The *inset* shows two Hu-positive, BrdU-labeled cells (one strongly and one weakly BrdU labeled). *D*, Approximately half of the transplanted cells (*arrowheads*) were double-labeled with NeuN in both the granule cell layer (*D*) and the periglomerular layer (*E*). *G*, Glomerulus. *Insets* in *D* and *E* show double-labeled cells in higher magnification. *F*, *G*, A small proportion of the BrdU-positive cells found in the periglomerular layer were also TH positive (*arrowheads*). Scale bar (shown in *A*): *A–F*, 100 μm; *G*, 50 μm.

Table 1. The properties of BrdU-labeled cells, which also express other markers of mature CNS phenotypes, at 6 weeks after transplantation to different regions of the adult rat brain

	SVZa and RMS transplants			
	Graft core	RMS	Granular layer	Periglomerular layer
Hu	0	+	++++	+++
β-tubulin-III	0	+	−	−
NeuN	0	0	+++	++
GAD_{67}	0	0	+++	++
TH	0	0	−	+
DARPP-32	0	0	0	0
GFAP	0	0	0	0
Vimentin	0	0	0	0

	Hippocampal transplants				
	Graft core	Subgranular layer	Granule cell layer	Hilus	CA3
Hu	++	+++++	++++	+	0
β-tubulin-III	++	+++++	++++	−	0
NeuN	+	+++++	++++	0	0
GAD_{67}	−	0	0	−	−
TH	0	0	0	0	0
DARPP-32	0	0	0	0	0
Calbindin	+	+++++	++++	0	0
GFAP	++	0	0	++	++

	Striatal transplants		
	Graft core	<0.4 mm from core	0.4–1.5 mm from core
Hu	+++++	++++	0
NeuN	0	0	0
GAD_{67}	+++	++	+
TH	0	0	0
DARPP-32	0	+	0
Calbindin	0	+,	0
GFAP	++	+++	+++

The frequency of double-labeled cells was quite consistent for the different phenotypic markers among the animals used for this analysis: olfactory bulb, $n = 8$; hippocampus, $n = 10$; striatum, $n = 10$. Double-labeling was assessed by confocal microscopy of randomly selected areas in sections stained with a combination of FITC-, Cy2-, and Cy3-labeled antibodies (see Materials and Methods). The data are based on observations from three representative animals in each group. 0, Cells not found; −, 1–10%; −+, 11–40%; +++, 41–60%; ++++, 61–80%; +++++, >80%.

The hippocampus

Transplants of 100,000 cells were placed within the hilar region of the dentate gyrus. At both 2 and 6 weeks after grafting, many of the injected BrdU-positive cells remained as a cluster just below the granule cell layer (Fig. 5*A*). This position of the cell deposit is characteristic for cells that are implanted by passive injection into the dentate gyrus, because of the presence of a cleavage plane underneath the granule cell layer (Wells et al., 1988). A significant proportion of the BrdU-positive cells, however, had migrated within the subgranular layer of the dentate gyrus and into the granule cell layer itself (Fig. 5*D–F*). In addition, some cells were found scattered in the hilus and the molecular layer of the dentate gyrus, as well as in the overlying CA3 region. The extent of cell migration was similar at 2 and 6 weeks. Typically, cells that had migrated longer distances from the transplant core were more weakly labeled with BrdU, suggesting that the migrated cells had undergone further cell divisions.

The BrdU-labeled cells that had integrated into the granular and subgranular layers had the same size and shape as the intrinsic host granule cells, and a large number of them expressed the neuronal markers Hu (Fig. 5*B*), NeuN (Fig. 5*C*), and β-tubulin-III (Fig. 5*D*) at both time points. The calbindin marker that is characteristic for the intrinsic granule cells was clearly present in many of the transplanted cells at 6 weeks but not at 2 weeks after transplantation. Occasional BrdU/Hu double-labeled cells, but no BrdU/NeuN or BrdU/calbindin double-labeled cells, were found outside these layers. A large proportion of the transplanted cells within the granule cell layer were calbindin positive (Fig. 5*E*). No BrdU/GAD_{67} double-labeled cells were observed in these transplants (Fig. 5*F*). Similarly, no cells that coexpressed BrdU and DARPP-32 were observed within any region of the hippocampus. BrdU-labeled cells expressing the glial marker GFAP were found in areas outside the dentate gyrus, both in the CA3 area and in areas close to ventricle as well as within or close to the graft core (see Fig. 9*C*). The extent of neuronal and glial differentiation of the transplanted cells within each region of the hippocampus is given in Table 1.

Staining with the hTau antibody revealed scattered axonal and cellular profiles, both within the graft core and in individual cells that had migrated away from the initial transplant site within the

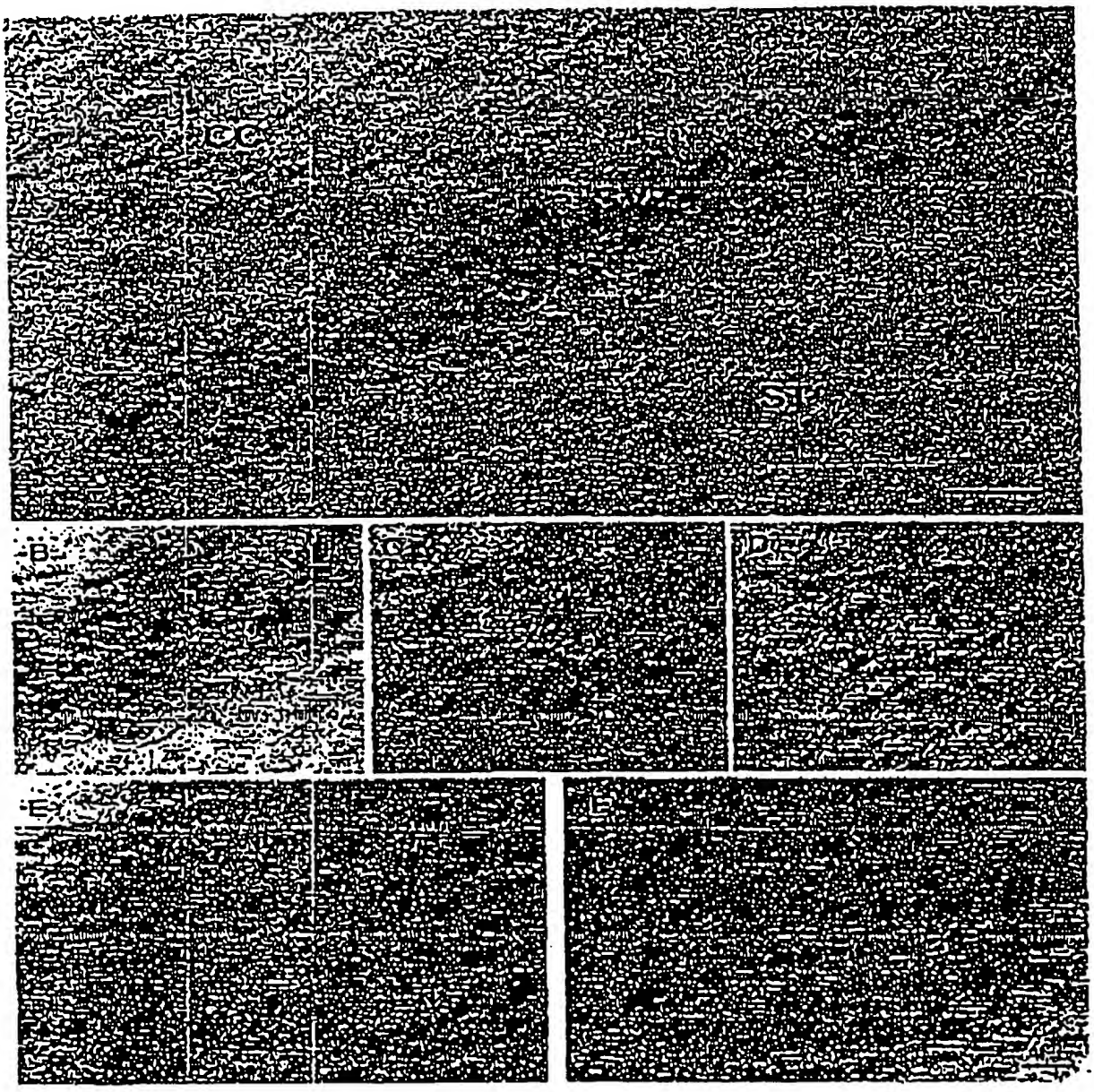

Figure 3. Grafts to the SVZa stained with a human-specific antibody to tau, at 6 weeks after transplantation. *A*, Sagittal section showing neuronal cell bodies and axons located within the SVZa, between the striatum (*ST*) and the overlying corpus calloqum (*CC*). *B–D*, Higher magnification of individual neuronal profiles at the periphery of the transplant (*B*) and migrating in the RMS (*C*, *D*). *E*, *F*, Individual cells located deep within the olfactory bulb, showing morphological features of mature neurons. Scale bars: *A*, 100 μm; (shown in *F*) *B–F*, 10 μm.

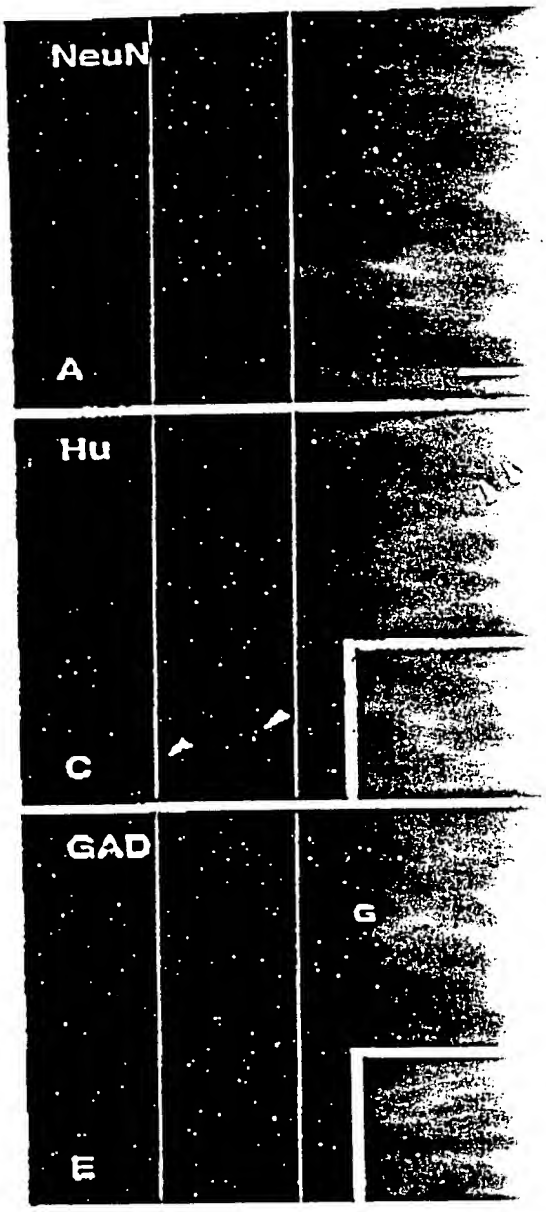

Figure 4. Confocal images of BrdU-labeled (*green*) and double-labe... transplantation, situated in the RMS with only moderate migration of ... after transplantation cells were seen migrating along the RMS. A sca... β-tubulin III (*arrowheads*). This marker was also present in many ... throughout the olfactory bulb. Many of them were Hu positive (*arro...* granule cell layer many of the BrdU-labeled cells were GAD_{67} positive ... GAD_{65} (*E*) or TH (*F*). *Insets* show individual double-labeled cells in ... (shown in *A*): *A*, 150 μm; *B*, *C*, 50 μm; *D–F*, 25 μm.

...ypical graft at 2 weeks after ... the graft core. *B*, At 6 weeks ...h the early neuronal marker ... cells were found scattered ... phenotype. *D*, Within the ...stained positively for either ... transplanted cell. Scale bar

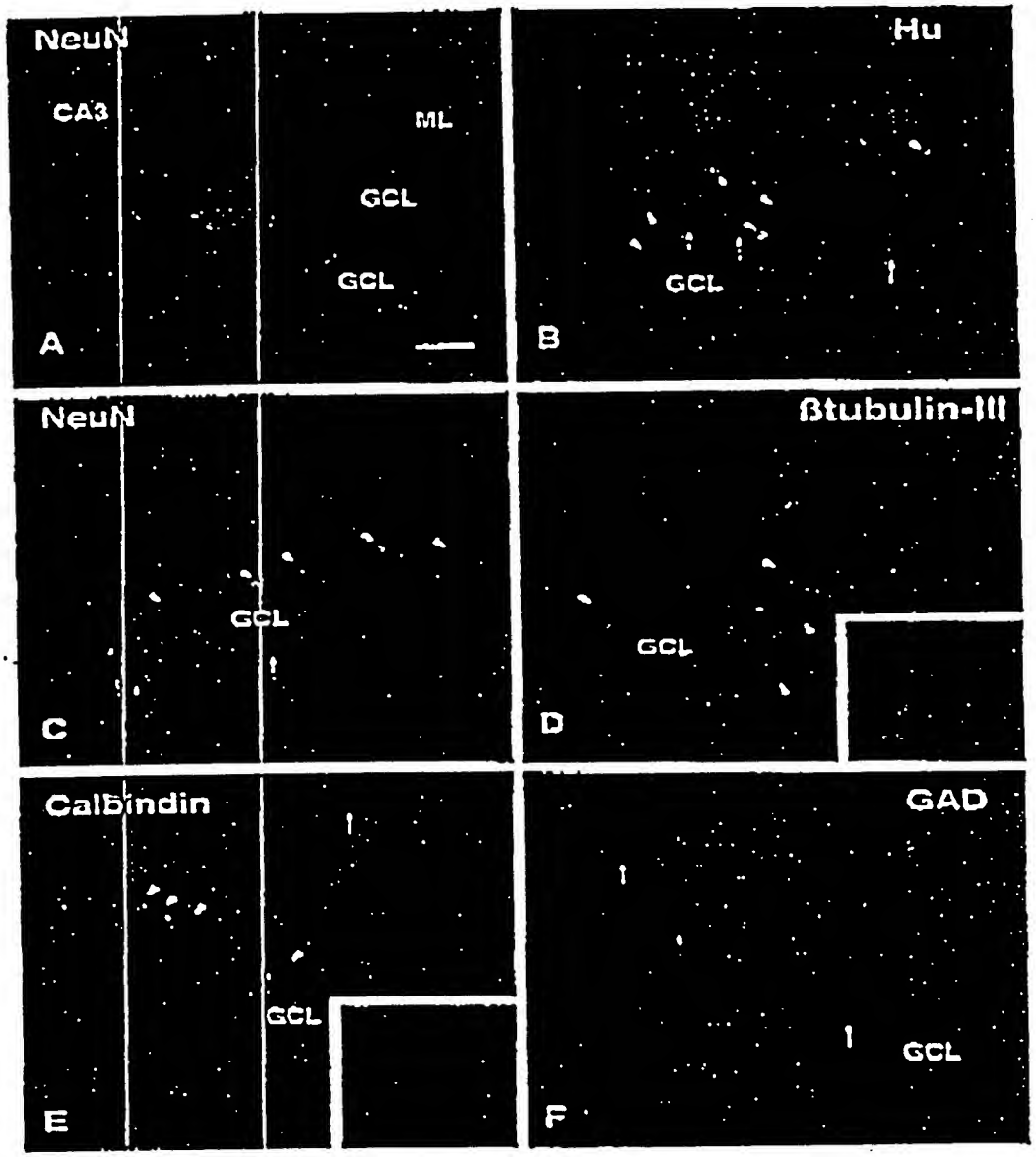

Figure 3. Confocal images of BrdU-labeled (*green*) and double-labeled cells (*yellow*) transplanted to the hippocampus. *A*, The core of transplanted cells was located within either the dorsal or ventral blades of the granule cell layer (*GCL*) in the dentate gyrus. *B*, *C*, By 2 weeks after transplantation, Hu- and NeuN-positive BrdU-labeled cells were observed at some distance from the graft core, mainly in the subgranular layer and also within the granule cell layer (*arrowheads*). *D*, BrdU-labeled transplanted cells positive for the neuronal marker β-tubulin-III (*arrowheads*) were found both within the graft core and in cells that had migrated along the subgranular layer. *E*, At 6 weeks (but not at 2 weeks) after transplantation, calbindin-positive cells were observed in the granule cell layer (*arrowheads*). *F*, No GAD$_{67}$-positive interneurons were observed. *Insets* show an individual β-tubulin-III/BrdU-labeled cell in the cluster of grafted cells in the subgranular layer in *D*, and two calbindin/BrdU-labeled cells within the deep part of the granule cell layer. *Arrows* indicate single-labeled BrdU-positive cells. Scale bar (shown in *A*): *A*, 150 μm; *B*, *C*, 75 μm; *D–F*, 30 μm. *CA3*, CA3 region of hippocampus; *ML*, molecular layer; *GCL*, granule cell layer.

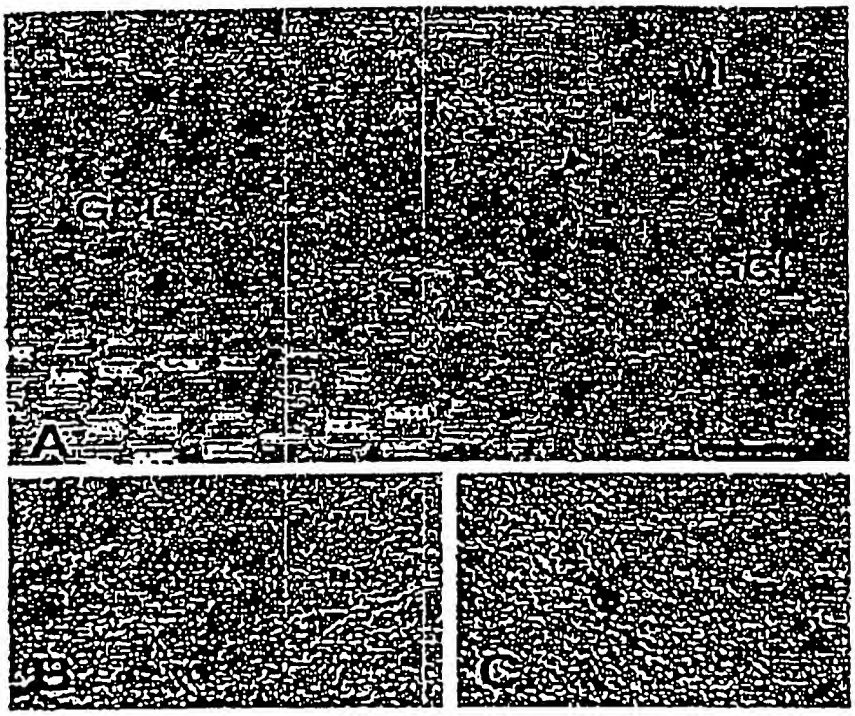

Figure 6. Hippocampal transplants stained with the hTau antibody. *A*, A transplant in the dentate gyrus at 6 weeks after transplantation. *B*, A tau-positive cell with a typically immature neuronal profile with one primary process. *C*, A more differentiated tau-positive neuron with more complex processes, situated within the subgranular layer (*arrowhead* in *A*). Scale bar (shown in *A*): *A*, 150 μm; *B*, *C*, 30 μm. *ML*, Molecular layer; *GCL*, granule cell layer; *H*, hilus.

granular and subgranular cell layers (Fig. 6*A*). At 2 weeks the cells appeared fairly immature, with a few short Tau-positive processes. At 6 weeks, cells with morphological features of neurons with processes were observed (Fig. 6*B*,*C*). Tau-positive cells were also seen in the hilus and molecular layer and along the needle tract.

The striatum

The transplants were placed centrally within the head of the caudate-putamen. At both 2 and 6 weeks after grafting, the grafted cells were found as a BrdU-labeled cell cluster at the site of implantation. Many of the BrdU-labeled cells, however, were observed to have migrated into the surrounding host striatum, without any preferential direction, to a distance of ~1–1.5 mm from the graft core (Fig. 7*A*). The size of individual BrdU nuclei varied considerably, both within the graft core and in cells that were located in the adjacent host striatum (<0.4 mm from the graft core). All of the cells that had migrated over longer distances were of small size and more faintly labeled, suggesting a dilution of the BrdU label caused by cell division. In sagittal sections the BrdU-positive cells could be seen to be aligned with the gray matter, interspersed with the fibers of the internal capsule.

Double-staining revealed that the majority of the BrdU-positive cells in the graft core and in the adjacent host striatum were double-labeled for the early neuronal marker Hu (Fig. 7*B*) but negative for NeuN (Fig. 7*C*). Many BrdU/Hu double-labeled cells occurred also at the graft-host border and within the adjacent host striatum, up to a distance of ~0.3–0.4 mm from the graft core. Although the majority of the Hu-positive cells within the graft core were small in size and round or oval in shape, similar to the Hu-positive cells within the SVZ of the host brain, a substantial proportion of the BrdU/Hu-positive cells at the graft-host border and in the host striatum were larger in size (10–15 μm), i.e., in the range of the Hu-positive neurons within the host striatum. None of the cells expressed NeuN, which is also the case, however, for most of the host striatal neurons. All BrdU-labeled cells located farther away from the graft core were Hu negative. These cells were all of small size and often found in satellite positions, closely apposed to host striatal neurons (Fig. 7*C*, *arrows*) or close to blood vessels. The location and staining properties of these small-sized cells suggest that they had differentiated, at least in part, into glia. Colocalization of BrdU and the astrocyte marker GFAP was unequivocally demonstrated at the graft-host border, i.e., within the area of GFAP-positive reactive astrocytes surrounding the graft core (see Fig. 9*D*, *inset*).

The neuronal phenotype of the transplanted cells was further investigated using antibodies against the GABA-synthesizing enzyme GAD_{67}, which is present in the vast majority (>90%) of the neurons within the striatum; DARPP-32, which is a marker for the medium-sized spiny striatal projection neurons; and calbindin, which is normally present in the medium spiny projection neurons in the matrix component of the striatum (for review, see Gerfen, 1992). BrdU/GAD_{67} double-labeled cells were observed both in the transplant core and within the host striatum at the

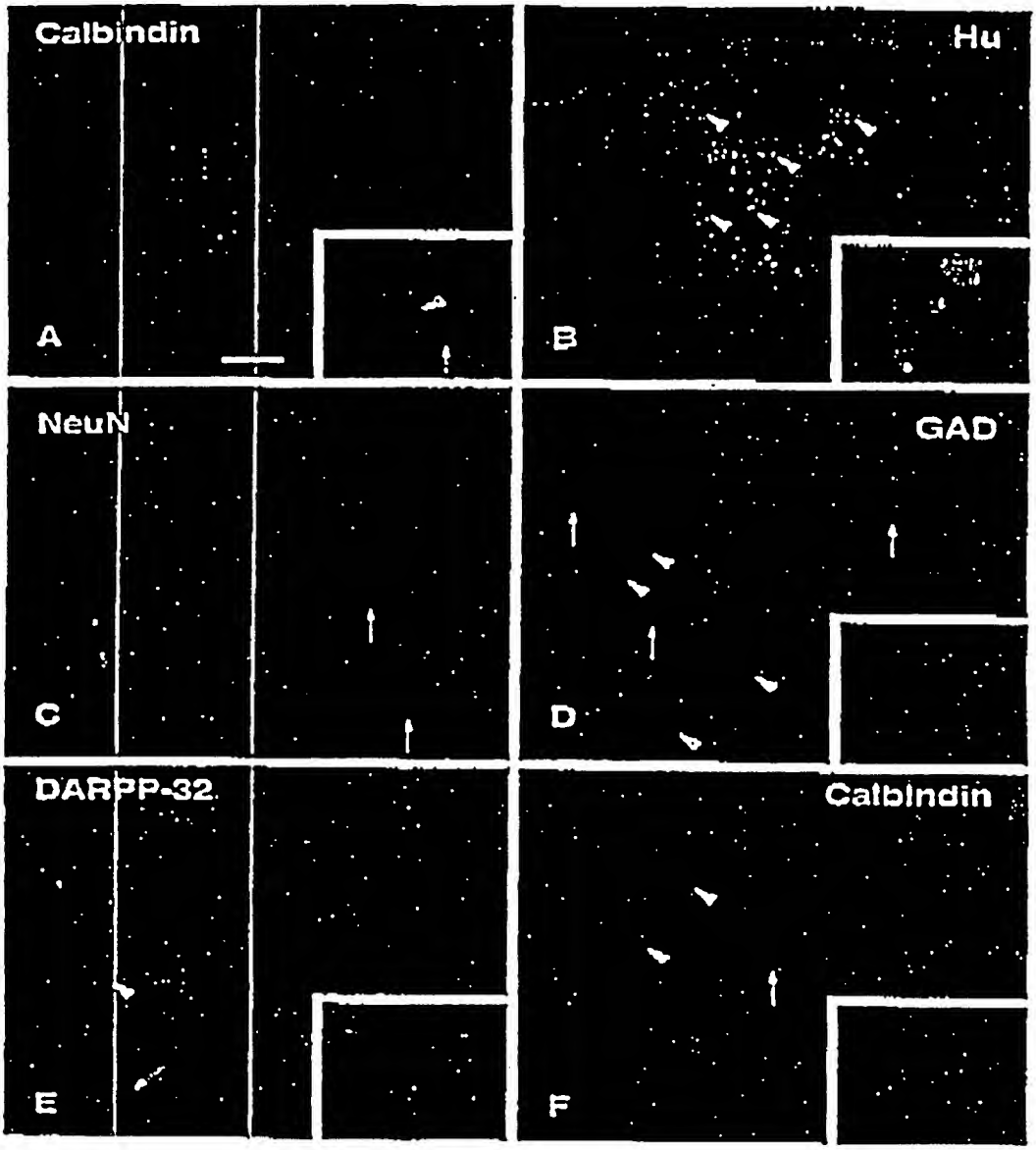

Figure 7. Confocal images of BrdU-labeled (*green*) and double-labeled cells (*yellow*) transplanted in the striatum. *A*, Coronal section through the graft core at 6 weeks after transplantation, showing a dense cluster of cells at the injection site and migration of BrdU-labeled cells away from the graft core. In both gray and white matter, *inset* shows region in *box* at higher magnification, also illustrated in *F*. *B*, Many of the transplanted cells were positively stained with Hu (*red*), even within the graft core (*arrowheads*, enlarged in the *inset*). *C*, No BrdU/NeuN double-labeled cells were found in the graft core or among those cells that had migrated into the host striatum. *Arrows* indicate transplanted cells that were found in close association with NeuN-positive host neurons (*red*). *D*, A number of transplanted cells were positive for the enzyme GAD_{67} in the periphery of the graft core (*arrowheads*). One of the double-labeled cells is shown at higher magnification in the *inset*. *E*, BrdU/DARPP-32 double-labeled cells were occasionally observed (*arrowhead* and *inset* at higher magnification). These were generally faintly labeled and found only in the immediate vicinity of the transplant core. *F*, Similarly, BrdU/Calbindin double-labeled cells were found both in the periphery of the graft and in adjacent regions of the host striatum. Scale bar (shown in *A*): *A*, 300 μm; *B*, 150 μm; *D–F*, 25 μm.

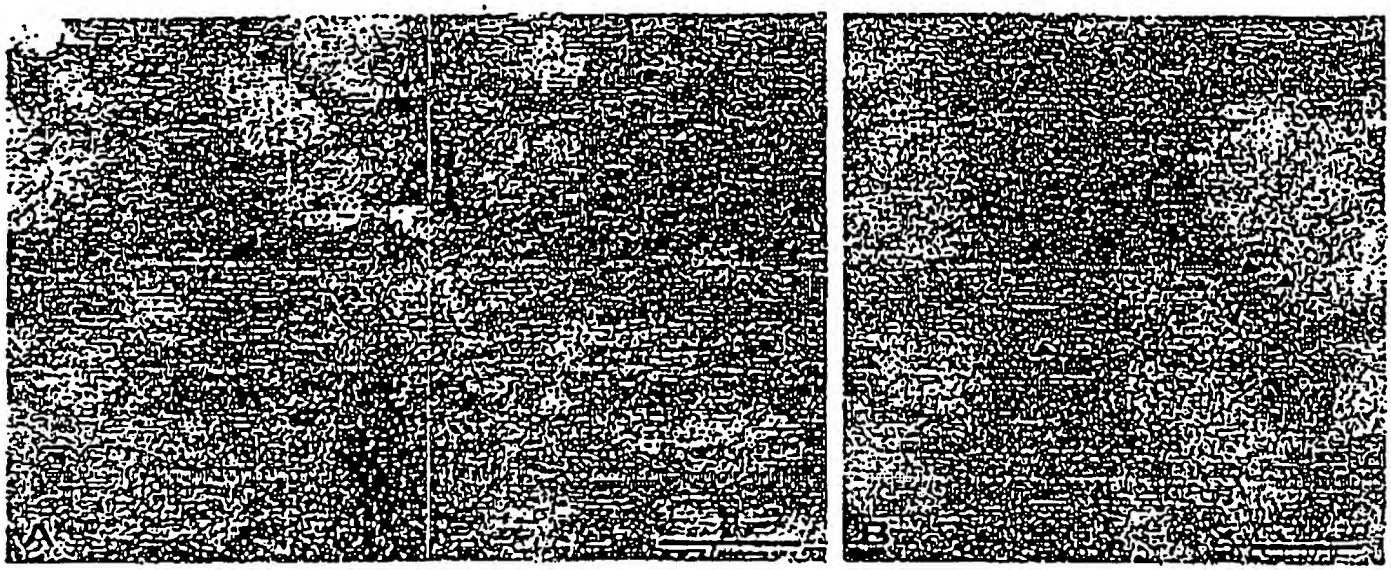

Figure 8. Striatal transplants stained with the hTau antibody. Six weeks after transplantation, coronal sections revealed tau-positive neuronal profiles densely packed within the graft core (*A*). Individual cells with neuronal profiles were observed also in the host striatum adjacent to the graft (*arrowheads* in *A* and *B*). Axonal processes were seen to extend caudally within the white matter bundles of the internal capsule (*arrows* in *A* and *B*). Scale bars: *A*, 500 μm; *B*, 100 μm.

periphery of the transplants (Fig. 7*D*). In addition, some BrdU-labeled cells expressed calbindin (Fig. 7*A,F*, *inset*) and occasionally also DARPP-32. These cells were located at the periphery of the transplants and in the adjacent host striatum up to a distance of ~0.3–0.4 mm from the graft-host border and were similar in size and shape to those present within the host striatum. The BrdU/DARPP-32 double-labeled cells were only weakly DARPP-32 positive but were comparable in size to the host DARPP-32-positive neurons (Fig. 7*E*). None of the transplanted cells expressed TH, either within the graft core or within the host striatum. Table 1 outlines the extent of expression of neuronal and glial markers at different distances from the graft core.

Staining with the hTau antibody revealed a graft core of clustered tau-positive cells and fibers (Fig. 8*A*). In sagittal sections, loose bundles of tau-positive fibers were seen to leave the graft core in both the rostral and caudal direction, along the white matter bundles of the internal capsule. In cross section, these fibers were found primarily within the white matter bundles (Fig. 8*B*, *arrows*). Individual cells were also observed at some distance from the graft core (Fig. 8*A,B*, *arrowheads*). In those cases, the cell bodies were often located within the gray matter, with their processes projecting into the white matter tracts. At 6 weeks, tau-positive axons could be traced caudally from the graft core within the internal capsule bundles for a distance of ~1–2 mm; some of these fibers were seen to enter the globus pallidus, and in some cases scattered tau-positive fibers could be traced as far as the entopeduncular nucleus.

DISCUSSION

These present results show that the long-term propagated human neurosphere cultures contained progenitors that can respond *in vivo* to cues present in both neurogenic and non-neurogenic regions of the adult rat brain. The expression of phenotypic markers provided evidence for site-specific neural differentiation within each of the three grafted regions. In the olfactory bulb the cells that integrated into the granular and periglomerular layers expressed NeuN, TH, and GAD_{67}, similar to the dopaminergic and GABAergic cells normally present in these regions. In the dentate gyrus some of the cells assumed a position, morphology, and phenotype similar to the NeuN/calbindin-positive granule cells within the granule cell layer. And in the striatum, cells located in the periphery of the transplants expressed GAD_{67} and calbindin as well as low levels of the striatum-specific marker DARPP-32.

A combination of EGF, bFGF, and LIF was used to expand the human progenitors. It has been shown previously that EGF and bFGF act cooperatively in promoting the proliferation of rat and human neural progenitors (Vescovi et al., 1993; Weiss et al., 1996a; Svendsen et al., 1997). bFGF appears to be a mitogen for both unipotent and multipotent neuronal and glial progenitors (Murphy et al., 1990; Vescovi et al., 1993; Ray and Gage, 1994; Kilpatrick and Bartlett, 1995; Palmer et al., 1995) and may act broadly to maintain neural progenitor cells as a constitutively proliferating population *in vitro* (Palmer et al., 1995). It seems likely, therefore, that the combination of growth factors used here served to maintain both multipotent and lineage-restricted progenitors in continuous cell cycle and that the ability to migrate and integrate into the adult host brain was expressed by specific subsets of cells. Previous studies suggest that the *in vivo* properties of *in vitro* expanded neural progenitors may differ depending on the growth factors used. Rat or mouse neurosphere cells ex-

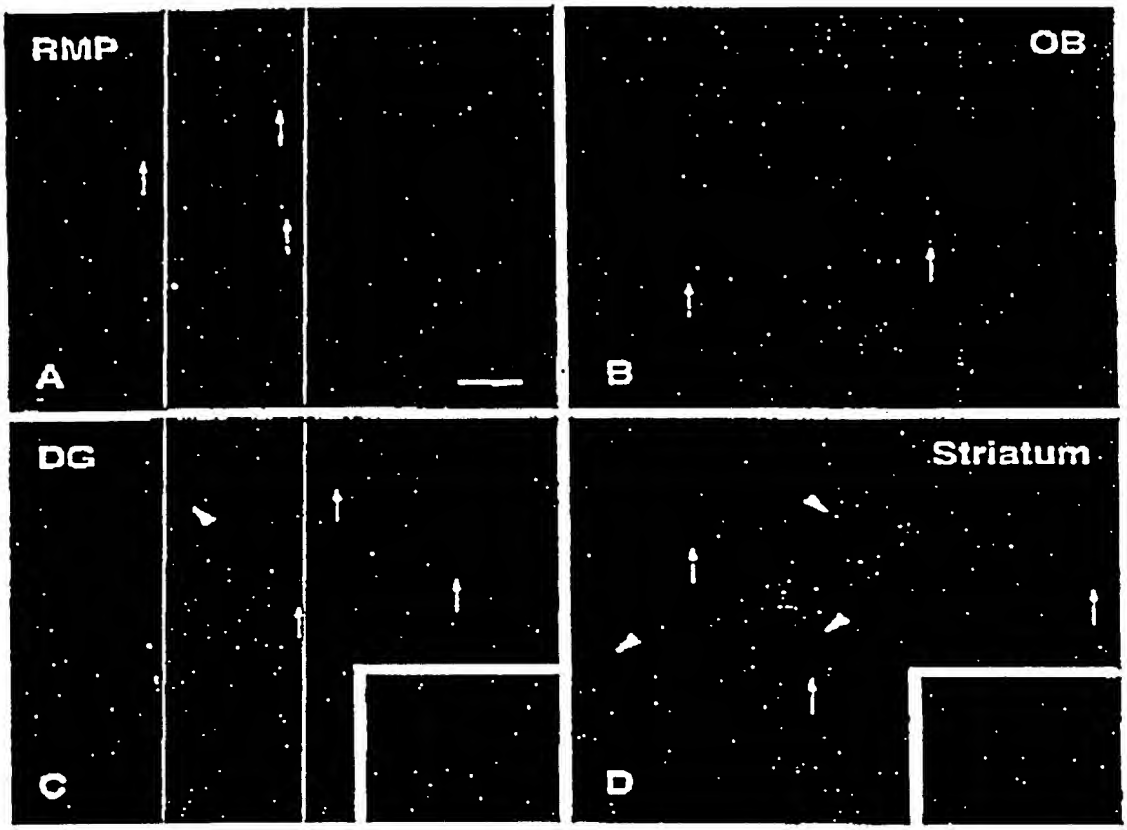

Figure 9. GFAP was used to label astrocytes within the graft areas (*red*) to assess the extent of colocalization with BrdU-labeled transplanted cells (*green*). *A*, Cells within the RMS at 6 weeks after transplantation were often closely associated with GFAP profiles, although no double-labeled cells were observed (*arrows*). *B*, Within the granule cell layer of the olfactory bulb, BrdU-positive cells were interspersed with, but not colocalized with, GFAP (*arrows*). *C*, In the dentate gyrus, cells within the transplant core were occasionally closely associated with GFAP-positive cytoplasmic staining, possibly indicating a double-labeled cell. *D*, Staining within the striatum revealed a dense network of GFAP-positive processes (*red*) intermingled with the BrdU-positive cells (*green*). Many clear examples of double-labeled cells were observed (*arrowheads*), although examples of BrdU single-labeled cells were also frequently observed (*arrows*). *Insets* show BrdU/GFAP double-labeled cells at higher magnification. Scale bar (shown in *A*): *A*, *C*, 50 μm; *B*, 25 μm; *D*, 150 μm.

pended in the presence of EGF alone have generated only glial cells and no neurons after transplantation to the developing rat forebrain (Winkler et al., 1998) or adult rat spinal cord (Hammang et al., 1997), and they exhibit poor survival and integration after transplantation to the striatum (Svendsen et al., 1996; C. Winkler, R. A. Fricker, A. Björklund, unpublished observations). By contrast, adult rat hippocampal progenitors cultured in the presence of bFGF exhibit both migration and neurogenesis after transplantation in the adult rat brain (Osga et al., 1995; Suhonen et al., 1996).

Site-specific differentiation of the grafted cells

In the SVZa, which is one of the two sites where neurogenesis continues into adulthood in the mammalian CNS, the endogenous neuronal progenitors have been shown to migrate along the RMS and reach the bulb within 2–15 d after their generation in the SVZa (Lois and Alvarez-Buylla, 1994). The cells are already committed to a neuronal phenotype while in the migratory path, although they continue to divide during migration (Menezes et al., 1995). The cells generated by SVZa postnatally are interneurons, above all GABAergic and dopaminergic interneurons in the granular and periglomerular layers of the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Betarbet et al., 1996).

The transplanted human neural progenitor cells expressed the early neuronal markers Hu and β-tubulin-III during migration to the olfactory bulb, indicating that some of the transplanted progenitors were committed to a neuronal fate already in the SVZ, similar to the endogenous neuronal progenitors generated in the SVZa (Lois and Alvarez-Buylla, 1994; Menezes et al., 1995). On

reaching the bulb, BrdU-positive cells distributed in the granular and periglomerular layers and coexpressed neuronal markers such as Hu and NeuN, as well as hTau. This is in agreement with previous results obtained with rat or mouse SVZa progenitors (Luskin, 1993; Lois and Alvarez-Buylla, 1994) and a recent study using transplantation of human neural stem cells (Flax et al., 1998). One interesting difference between the transplanted human cells in the current study and endogenous SVZa progenitors is the time course of migration: few of the transplanted human progenitors had entered the RMS at 2 weeks, and many still remained dispersed along the RMS by 6 weeks. One reason for this may be a species difference. Transplants of human primary cells show a more protracted development than rat-to-rat grafts, which suggests that the human cells retain some type of internal developmental clock for their differentiation and maturation (Grasbon-Frodl et al., 1996, 1997). Indeed, Suhonen et al. (1996) reported that adult rat neural progenitors transplanted to the SVZa in adult rats are distributed along the entire length of the RMS by 1 week, and by 8 weeks ~90% of the cells had reached the bulb. Similarly, Lois and Alvarez-Buylla (1994) observed that SVZa progenitors, implanted into the adult SVZa, reach the bulb within 30 d after transplantation. These observations indicate that the slow onset and protracted time course of migration of the human cells reflect intrinsic developmental constraints.

In hippocampus the transplanted cells distributed along the subgranular and granular layers of the dentate gyrus. Although cells were observed also in other layers of the dentate and the CA3 region, cells expressing neuronal markers occurred only within the subgranular or granular layers, suggesting that the human progenitors, similar to rat hippocampal and cerebellar progenitors (Gage et al., 1995; Vicario-Abejon et al., 1995), are able to respond to local cues specifically localized in these layers. The transition zone between the hilus and the granule cell layer is the site where endogenous neuronal progenitors are normally generated (Altman and Das, 1966; Altman and Bayer, 1990), providing a source of new granule cells throughout life (Kaplan and Hinds, 1977; Cameron et al., 1993; Kuhn et al., 1996). As judged by morphological criteria, i.e., size, shape, and distribution of the cells, and expression of characteristic neuronal markers, the grafted progenitors are induced by local signals to express neuronal features similar to the resident granule cells. It remains to be demonstrated, however, to what extent these newly formed neurons can undergo complete maturation and establish appropriate axonal and dendritic connectivity.

Cells grafted to the striatum generate both neurons and glia

Expression of neuronal markers in the striatal transplants indicate that a substantial fraction of the grafted human progenitors had developed toward a neuronal phenotype. Many of the Hu-positive cells within the transplant core were small and round or oval in shape, similar to the neuronal precursors normally present in the proliferative subependyma in the adult brain. These cells did not express any of the more mature neuronal markers and therefore may be classified as poorly differentiated neuronal precursors. The GAD_{67}-, calbindin-, and DARPP-32-positive cells were exclusively located at the graft-host border and within the adjacent host striatum, up to a distance of ~0.3–0.4 mm. The size and shape of these cells were similar to the medium-sized neurons of the host striatum. Many of these are GABAergic and stain positively for GAD_{67}; one subclass, the striatal projection neurons, is further characterized by the expression of calbindin and/or DARPP-32.

These data indicate that the human neural progenitors can undergo neurogenesis also in the site normally non-neurogenic environment of the adult striatum and assume neuronal phenotype(s) similar to those normally present here but that in the absence of suitable substrates for migration they remain close to the implantation site. Interestingly, in sections stained with the hTau antibody some of these newly formed neurons were seen to extend long axon-like processes that could be traced along the fascicles of the internal capsule to the globus pallidus and in some cases also the entopeduncular nucleus, a distance of ~2 mm.

The cells that migrated over longer distances within the adult striatum were all Hu negative and of small size. Many of them were found in satellite position to the medium-sized host striatal neurons or close to blood vessels, suggesting that they had assumed a glial-like phenotype (Fig. 9). A migratory capacity of immature glia (or glial precursors) within the adult CNS has been reported for both astrocytes and oligodendrocytes by several investigators (Blakemore and Franklin, 1991). Extensive astrocyte migration within the adult striatum, similar in extent to the one observed here, has previously been described in transplants of human neuronal progenitors (Svendsen et al., 1997) and freshly dissociated human embryonic striatal and dienchephalic tissue (Pundt et al., 1995). In these cases the migratory cells appear to be glial precursors in a proliferative, migratory stage of their development. Consistent with this, we observed that cells located at progressively greater distances from the transplant core had lower levels of BrdU labeling than the cells that remained at the implantation site, suggesting that the migrating cells continued to divide as they dispersed within the host striatal parenchyma.

Implications for brain repair

The human neurosphere cultures are particularly suitable for transplantation in that they can be harvested and implanted without dissociation and detachment from a culture substrate. The cultures used here had been expanded up to 10 million-fold, which means that each transplant of 100,000–200,000 cells in theory could be derived from a single cell in the original cell preparation. Because the *in vivo* properties of the cells were indistinguishable over a wide range of passages (from 9 to 21), the present culture system could provide an almost unlimited source of human neural progenitor cells for transplantation.

The present results show that subpopulations of cells contained within the human neurosphere cultures can respond appropriately to specific extracellular cues present in each of the four target regions in the adult rat brain. Because the human neurosphere cultures are likely to contain a mixture of multipotent and lineage-restricted progenitors, the specific migratory patterns seen in the different locations may be explained either by the ability of an undifferentiated stem cell-like cell to differentiate along alternative neuronal or glial pathways in response to diverse local cues, or alternatively, by the presence of different subpopulations of lineage-restricted neuronal or glial precursors that were already committed to specific neuronal or glial fates. The present data seem compatible with both alternatives.

In conclusion, the long-term propagated human neural progenitors described here demonstrate a remarkable capacity for migration, integration, and site-specific differentiation in the adult brain. The growth factor combination used here acted to maintain the progenitors as a constitutively proliferating cell popula-

tion without losing their capacity to respond to those extracellular cues normally present in the adult CNS. With further refinement of the procedure, e.g., by application of cell enrichment and cell sorting techniques, this culture system may provide an almost unlimited source of human neural progenitors at different stages of differentiation and lineage restriction. Such cells will be of great interest both as an experimental tool and as an alternative to primary embryonic brain tissue for intracerebral transplantation.

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Neural stem cells display extensive tropism for pathology in adult brain: Evidence from intracranial gliomas

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One of the impediments to the treatment of brain tumors (e.g., gliomas) has been the degree to which they expand, infiltrate surrounding tissue, and migrate widely into normal brain, usually rendering them "elusive" to effective resection, irradiation, chemotherapy, or gene therapy. We demonstrate that neural stem cells (NSCs), when implanted into experimental intracranial gliomas *in vivo* in adult rodents, distribute themselves quickly and extensively throughout the tumor bed and migrate uniquely in juxtaposition to widely expanding and aggressively advancing tumor cells, while continuing to stably express a foreign gene. The NSCs "surround" the invading tumor border while "chasing down" infiltrating tumor cells. When implanted intracranially at distant sites from the tumor (e.g., into normal tissue, into the contralateral hemisphere, or into the cerebral ventricles), the donor cells migrate through normal tissue targeting the tumor cells (including human glioblastomas). When implanted outside the CNS intravascularly, NSCs will target an intracranial tumor. NSCs can deliver a therapeutically relevant molecule—cytosine deaminase—such that quantifiable reduction in tumor burden results. These data suggest the adjunctive use of inherently migratory NSCs as a delivery vehicle for targeting therapeutic genes and vectors to refractory, migratory, invasive brain tumors. More broadly, they suggest that NSC migration can be extensive, even in the adult brain and along nonstereotypical routes, if pathology (as modeled here by tumor) is present.

gene therapy | transplantation | migration | brain tumors | vascular

Malignant brain tumors, e.g., glioblastoma multiforme, remain virtually untreatable and inevitably lethal despite extensive surgical excision and adjuvant radio- and chemotherapy (1). Their treatment resistance is related to their exceptional migratory nature and ability to insinuate themselves seamlessly and extensively into normal neural tissue, often migrating great distances from the main tumor mass. These cells are responsible for the recurrent tumor growth near the borders of the resection cavity (1). It is this behavior that has also limited their accessibility to otherwise promising gene therapeutic vectors and interventions (2). Intriguingly, one of the cardinal features of neural stem cells (NSCs) is their exceptional migratory ability (3–10). Indeed, it is their migratory capacity that has made them so useful in therapeutic paradigms demanding brainwide gene and cell replacement in various animal models of neurodegeneration, albeit usually in the newborn (8–10). We hypothesized that pathology promotes NSC migration to an extent not assumed possible based on knowledge drawn from the normal adult brain and that, therefore, an approach for targeting gene therapy to the most migratory tumor cells in the adult central nervous system (CNS) might be the use of inherently migratory NSCs to deliver therapeutic genes and/or their products.

Experimental Methods

***In Vitro* Migration Studies.** CNS-1 is a virulent, invasive rat-derived glioblastoma cell line. Cells engineered to express green fluorescent protein (GFP) as previously described (11, 12) were plated to 60–70% confluence onto 100-mm culture dishes around a central 5-mm metal cylinder that was sealed and, therefore, remained cell-free. The plate was incubated overnight, by which time the glioma cells had attached. A suspension of 4×10^4 dissociated fibroblasts (in control dishes) or murine NSCs (in experimental dishes) were seeded into the central cylinder (i.e., no direct contact with CNS-1 cells) (Fig. 1, arrowheads). A similar number of fibroblasts or NSCs, respectively, were placed into a 5-mm cylinder placed directly on top of the adherent CNS-1 monolayer and cultured as before (at extreme right edge of plates) (Fig. 1, arrows). The fibroblasts (clone TR-10) were derived from 3T3 cells infected with a retroviral vector encoding *lacZ*. The murine NSCs were derived from the prototypical constitutively *lacZ*-expressing helper virus-free murine NSC clonal line C17.2 (8–10, 13), which, because of its well-documented ability to integrate into most CNS structures and in a number of normal and abnormal animal models, has been useful for delineating the range of therapeutic possibilities for NSCs (8–10, 13). (Although self-renewing, NSCs become contact inhibited; never grow in soft agar; are nontumorigenic in nude mice; fail to incorporate BrdUrd after 48 h *in vivo*; and respond to normal cues for cell cycle withdrawal, differentiation, and interaction with host cells.) The day after plating of fibroblasts and NSCs, the cylinders were removed, and the dishes were rinsed and incubated for an additional 5 days. Dishes were stained for the *lacZ* gene product *Escherichia coli* β-galactosidase (β-gal) by 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) histochemistry (8).

Animal Studies *in Vivo*. For some studies, not only were murine NSCs (C17.2) used but also some of human derivation were used (5). To establish intracranial tumors, either the CNS-1 rat glioblastoma line (11, 12) or the HGL21 human glioblastoma line (14) was implanted into the brains of adult female nude mice, or the D74 glioma line was implanted into the brains of adult female Fisher rats by using procedures previously described (15). Briefly, animals received stereotactically guided injections over 3–5 min into the forebrain (2 mm lateral and 1 mm anterior to bregma; depth 2–4 mm from dura) of tumor cells (of a number specified below) suspended in 1 μl of PBS. Animals receiving a second implant at a later date of NSCs or fibroblasts [suspended in PBS at $2–4 \times 10^4$ cells per μl as detailed elsewhere (8–10, 13)] were injected stereotactically with cells in a quantity and location to yield the various paradigms described below. On

Abbreviations: NSC, neural stem cell; CNS, central nervous system; GFP, green fluorescent protein; β-gal; β-galactosidase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; CD, cytosine deaminase; 5-FC, 5-fluorocytosine; CD-NSC, CD-transduced NSC.

See commentary on page 12393.

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Fig. 1. Migratory capacity of NSCs in culture. CNS-1 glioblastoma cells were plated around a central cylinder (i.e., free of CNS-1 cells). Fibroblasts (*A*) or NSCs (*B*) were seeded into the center cylinder (i.e., no direct contact with CNS-1 cells) (arrowheads) or into cylinders placed directly on top of adherent tumor cells (at the extreme right edge of plates; arrows). After removal of the cylinders and 5 additional days of incubation, there was wide distribution of blue X-Gal$^+$ NSCs (*B*), compared with fibroblasts (*A*), which remained localized to their area of initial seeding.

the days specified below, the brains were processed as detailed below (e.g., for β-gal, GFP, BrdUrd, human markers, and/or cell type-specific antigen expression). CD-1 mice, when used, received daily cyclosporin injections (10 μg/g).

Specific Protocols for *in Vivo* Experiments. *NSC implantation directly into tumor bed (Fig. 2, Paradigm 1).* On day 0, recipients received injections of tumor cells (3–4 × 10^4 in 1 μl of PBS) into the right frontal lobe. On day 4–6, NSCs or fibroblasts (4–10 × 10^4 in 1.5 μl of PBS) were injected directly into the tumor bed, using identical coordinates. Recipients were killed on days 6–9, 10–12, 14–16, and 21 after tumor implantation.

NSC implantation at distant intracranial site from tumor bed: In same hemisphere (Fig. 3, Paradigm 2). On day 0, recipients received injections of glioblastoma cells (3 × 10^4 in 1 μl of PBS) into the right frontal lobe. On day 6, NSCs (4 × 10^4 in 1.5 μl of PBS) were injected also into the right frontoparietal lobe at the following coordinates: 3 mm lateral and 4 mm caudal to bregma; depth 3 mm from dura—i.e., 1 mm lateral and 4 mm *behind* the tumor. Animals were killed on days 12 and 21.

In contralateral hemisphere (Fig. 3, Paradigm 3). On day 0, recipients received injections of glioblastoma cells (3–5 × 10^4 in 1 μl of PBS) into the right frontal lobe (2.5 mm lateral and 2 mm caudal to bregma; depth 3 mm from dura). On day 6, NSCs (8 × 10^4 in 2 μl of PBS) were injected into the *left* frontal lobe at the following coordinates: 2 mm lateral and 2 mm caudal to bregma; depth 3 mm from dura. Animals were killed on days 12 and 21.

Into ventricles (Fig. 3, Paradigm 4). On day 0, recipients received injections of glioblastoma cells (5–8 × 10^4 in 1 μl of PBS) into the right frontal lobe (2 mm lateral to bregma on the coronal suture; depth 3 mm from dura). On day 6, NSCs (8 × 10^4 in 2 μl of PBS) were injected into the contralateral or ipsilateral cerebral ventricle at the following coordinates on the respective side: 1 mm lateral and 3 mm caudal to bregma; depth 2 mm from dura. Animals were killed on days 8, 12, and/or 21.

NSC implantation into a peripheral, intravascular site (Fig. 4, Paradigm 5). On day 0, adult nude mice received injections of CNS-1 glioblastoma cells (1 × 10^5 in 2 μl of PBS) into the right frontal lobe. On day 7, murine NSCs (2 × 10^6 in 200 μl of PBS) were injected into the tail vein. Animals were killed on day 12.

Retroviral Transduction of NSCs with Cytosine Deaminase (CD). A plasmid using the retroviral pBabePuro backbone (16) was constructed to include the *E. coli* CD cDNA (1.5-kb fragment) transcribed from the long terminal repeat. Vectors were packaged by cotransduction of the CDpuro plasmid with the MV12 envelope coding plasmid cDNA (17) into 293T/17 cells (18). CDpuro retroviral supernatant was used for multiple infections of the murine NSCs. Transduced NSCs ("CD-NSCs") were placed under puromycin selection for ≈2 wk.

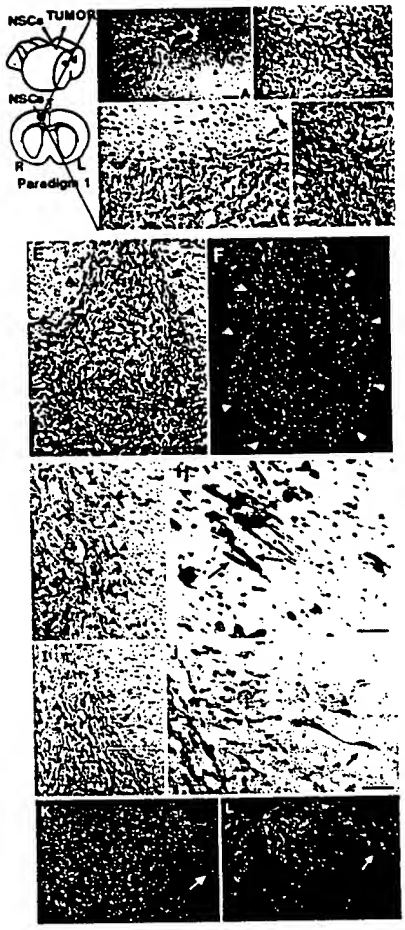

Fig. 2. NSCs migrate extensively throughout a brain tumor mass *in vivo* and "trail" advancing tumor cells. Paradigm 1 is illustrated schematically. Section of brain under low (*A*) and high (*B*) power from an adult rat killed 48 h after NSC injection into an established D74 glioma, processed with X-Gal to detect blue-staining β-gal-producing NSCs and counterstained with neutral red to show dark red tumor cells; arrowheads demarcate approximate edges of the tumor mass where it interfaces with normal tissue. Donor X-Gal$^+$ blue NSCs (arrows) can be seen extensively distributed throughout the mass, interspersed among the red tumor cells. (*C*) Tumor, 10 days after NSC injection, illustrating that, although NSCs (arrows) have infiltrated the mass, they largely stop at the junction between tumor and normal tissue (arrowheads) except where a tumor cell (dark red, elongated) is entering normal tissue; then NSCs appear to "follow" the invading tumor cell into surrounding tissue (upper right arrow). This phenomenon becomes more dramatic when examining NSC behavior in a more virulent and aggressively invasive tumor, the CNS-1 glioblastoma in the adult nude mouse, pictured in *D*. This section illustrates extensive migration and distribution of blue NSCs (arrows) throughout the infiltrating glioblastoma up to and along the infiltrating tumor edge (red arrowheads) and into surrounding tissue in juxtaposition to many dark red$^+$ tumor cells invading normal tissue. The "tracking" of individual glioblastoma cells is examined in greater detail in *E–L*, where CNS-1 cells have been labeled *ex vivo* by transduction with GFP cDNA. (*E* and *F*) Sister sections showing a low power view of transgene-expressing NSCs distributed throughout the main tumor mass to the tumor edge (outlined by arrowheads). Sections were either costained with X-Gal (NSCs, blue) and neutral red (tumor cells, dark red and elongated) (*E*) or processed for double immunofluorescence using an anti-β-gal antibody (NSCs, red) and an FITC-conjugated anti-GFP antibody (glioblastoma cells, green) (*F*). Low (*G*) and high (*H*) power views of tumor edge (arrowheads) with blue NSCs (blue arrow) in immediate proximity to and intermixed with an invading tumor "island" (dark red spindle-shaped cells) (red arrow). (*I* and *J*) Low and high power views, respectively (boxed area in *I* is magnified in *J*), of a blue NSC in direct juxtaposition to a single migrating neutral red$^-$, spindle-shaped tumor cell (arrow), the NSC "riding" the glioma cell in "piggy-back" fashion. (*K* and *L*) Low and high power views, respectively, under fluorescence microscopy, of single migrating GFP$^+$ tumor cells (green) in juxtaposition to β-gal$^+$ NSCs (red). Region indicated by white arrow in *K* and magnified in *L* illustrates NSCs apposed to tumor cells migrating away from the main tumor bed. (Scale bars: *A*, 40 μm, 30 μm in *B*; *C*, 30 μm, 25 μm in *D*; *E*, 90 μm, 100 μm in *F*; *H*, 15 μm, 60 μm in *G*; *J*, 30 μm, 60 μm in *I*, 70 μm in *K*, 35 μm in *L*.)

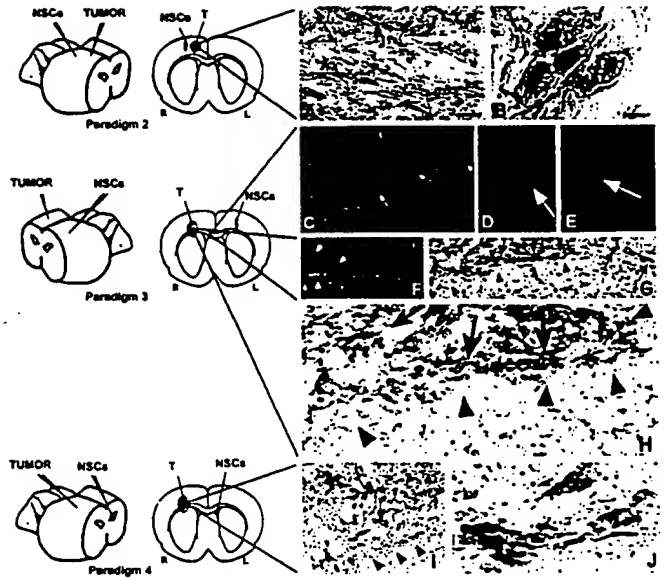

Fig. 3. NSCs implanted at various intracranial sites distant from main tumor bed migrate through normal adult tissue toward glioma cells. (*A* and *B*) Same hemisphere but *behind* tumor (Paradigm 2). Shown here is a section through the tumor from an adult nude mouse 6 days after NSC implantation caudal to tumor. In *A* (as per the schematic, a coned down view of a tumor populated as pictured under low power in Figs. 2*A* and 3 *A* and *B*), note X-Gal$^+$ blue NSCs interspersed among dark neutral red$^+$ tumor cells. (*B*) High power view of NSCs in juxtaposition to islands of tumor cells. (*C–H*) Contralateral hemisphere (Paradigm 3). (*C–E*) As indicated on the schematic, these panels are views through the corpus callosum ("c") where β-gal$^+$ NSCs (red cells, arrows) are seen migrating from their site of implantation on one side of the brain toward tumor on the other. Two representative NSCs indicated by arrows in *C* are viewed at higher magnification in *D* and *E*, respectively, to visualize the classic elongated morphology and leading process of a migrating neural progenitor oriented toward its target. In *F*, β-gal$^+$ NSCs (red) are "homing in" on the GFP$^+$ tumor (green) having migrated from the other hemisphere. In *G*, and magnified further in *H*, the X-Gal$^+$ blue NSCs (arrows) have now actually entered the neutral red$^+$ tumor (arrowheads) from the opposite hemisphere. (*I* and *J*) Intraventricular (Paradigm 4). Shown here is a section through the brain tumor of an adult nude mouse 6 days following NSC injection into the contralateral cerebral ventricle. In *I*, as per the schematic, blue X-Gal$^+$ NSCs are distributed within the neutral red$^+$ main tumor bed (edge delineated by arrowheads). At higher power in *J*, the NSCs are in juxtaposition to migrating islands of red glioblastoma cells. Fibroblast control cells never migrated from their injection site in any paradigm. All X-Gal-positivity was corroborated by anti-β-gal immunoreactivity. (Scale bar: *A*, 20 μm, and applies to *C*; *B*, 8 μm, 14 μm in *D* and *E*, 30 μm in *F* and *G*, 15 μm in *H*, 20 μm in *I*, and 15 μm in *J*.)

Oncolysis Assays of CD Bioactivity. For *in vitro* assays, CNS-1 cells (2×10^5) were plated onto 10-cm dishes (day 1). On day 2, murine or human CD-NSCs ($5–10 \times 10^4$) were added. On day 3, 5-fluorocytosine (5-FC, 500 μg/ml) was added. Control dishes included (*i*) cocultures with no 5-FC and (*ii*) tumor cells alone with 5-FC. On day 6, plates were stained by means of X-Gal histochemistry to visualize NSCs and with neutral red to visualize tumor cells (Fig. 6 *A* and *B*). The number of tumor cells was extrapolated from the average of 20 random high power fields per plate. For *in vivo* assays, animals bearing CNS-1 cells (7×10^4) alone or CNS-1 cells interspersed with CD-NSCs (3.5×10^4) received 10 i.p. injections of 5-FC (900 mg/kg) over 10 days. Control tumor-bearing animals received no 5-FC, 5-FC without NSCs, or NSCs without 5-FC. One day after the last 5-FC dose, the brains were cryosectioned and stained with X-Gal and neutral red, and measurements of the tumor were made from camera lucida drawings of the mass from interval sections through the tumor from which relative surface areas were then calculated by image analysis (Fig. 7).

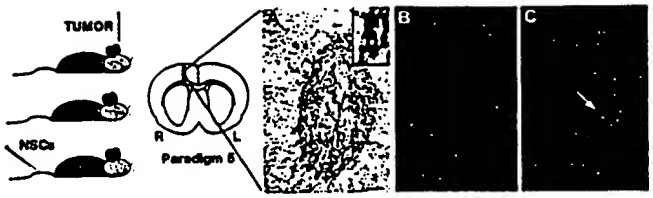

Fig. 4. NSCs injected into tail vein "target" intracerebral gliomas. Paradigm 5 is illustrated. (*A–C*) Progressively higher power views of representative 10-μm sections through the brain 4 days after NSC injection, processed with X-Gal histochemistry (*A*) and anti-β-gal immunocytochemistry (*B* and *C*) to identify donor NSCs and counterstained with neutral red to delineate the tumor border. (The β-gal immunoproduct, in addition to providing independent identity confirmation, typically fills cells and processes much better than X-Gal.) At low power (*A*), X-Gal$^+$ NSCs (representative X-Gal precipitate enlarged in *Inset*) are distributed throughout the tumor but not in surrounding normal tissue. Sister sections, reacted with an anti-β-gal antibody and visualized at higher power in *B* and further magnified in *C* confirm the presence of donor-derived cells (arrow) within the tumor. (Scale bar: *A*, 25 μm, 20 μm in *B*, and 12 μm in C.)

BrdUrd Uptake by Engrafted NSCs. Selected animals received three i.p. injections of BrdUrd (1 ml/100 g body weight of a 20 μM stock solution) over 24 h before sacrifice.

Histopathological and Immunocytochemical Analysis. On the days specified in the paradigms above, animals were killed, and 10- to 15-μm serial coronal cryosections from 4% paraformaldehyde-fixed brains were processed for light microscopy with X-Gal histochemistry to identify *lacZ*-expressing blue donor cells (8–10, 13) and counterstained with neutral red to detect distinctively dark red, elongated tumor cells (2). Sister sections were prepared for dual-filter immunofluorescence where an anti-β-gal antibody was revealed with a Texas Red-conjugated secondary antibody (1:1,000; Vectastain) (to identify donor cells as red) (8–10, 13), and an anti-GFP antibody (1:500; CLONTECH) was revealed with an FITC-conjugated secondary antibody (1:1,000; Vectastain) (to recognize CNS-1 tumor cells as green). BrdUrd$^+$-intercalated cells were identified by an anti-BrdUrd antibody (5). Sections containing human NSCs were additionally stained with multiple human-specific antibodies, including against to ribonuclear protein (Chemicon, 1:20), against NuMA (Chemicon, 1:40 and Calbiochem, 1:400), and against the human EGF receptor (Upstate Biotechnology, 1:100), revealed by a biotinylated goat anti-mouse IgM (1:250, Vectastain) followed by the standard ABC and diaminobenzidine (DAB) reaction (Vectastain).

Results

This study's focus was documenting the migratory behavior of NSCs [specifically those reported to be effective delivery vehicles for genes (5, 8) and viral vectors (10)] in relation to aggressively invasive experimental intracranial tumors in adult brain and then to arm some of these cells with a bioactive, therapeutic gene requiring relative proximity to tumor cells (allowing oncolysis to be a measure of the efficiency and specificity of gene expression).

Migratory Capacity of NSCs in Culture. To visualize the migratory properties of NSCs when confronted with a tumor, *in vitro* studies first assessed the relative migratory capacity of NSCs compared with fibroblasts cocultured with glioma cells. In contrast to fibroblasts, which remained localized to the area of initial seeding (Fig. 1*A*), NSCs migrated rapidly and interspersed throughout the glioma monolayer, far from their initial site of seeding, robustly expressing *lacZ* (Fig. 1*B*). These patterns were observed whether the cells were plated directly on top of (i.e., in direct contact with) the glioma cells (arrows) or merely within the same culture medium and environment without direct contact (arrowheads). The migra

ıg NSCs then became contact-inhibited and quiescent (do not ncorporate BrdUrd; ref. 5).

ISCs Migrate Throughout and "Surround" Tumor *in Vivo*. To deter-nine the behavior of NSCs introduced to brain tumors *in vivo*, adult ats first received an implant of syngeneic D74 rat glioma cells (19) nto the right frontal lobe. Four days later, *lacZ*-expressing murine ISCs were injected directly into the tumor bed (Paradigm 1, Fig.). Animals were killed at 2- to 4-day intervals after intratumoral njection and brain sections processed to detect β-gal-producing ISCs. As early as 2 days after injection, X-Gal$^+$ blue donor NSCs vere found distributed extensively throughout the darkly neutral ed-stained tumor mass (Fig. 2*A*). Although the transgene-xpressing NSCs remained stably intermixed throughout the tumor Fig. 2*B*, arrows), up to and along the infiltrating tumor edge, they argely stopped at the border of the tumor where it interfaced with ıormal tissue (Fig. 2*C*, arrowheads) as if "surrounding" the ad-ancing neoplasm. Normal adult parenchyma presented a less ermissive migratory environment to NSCs, except under one ircumstance: where tumor cells began to infiltrate normal brain. In hose instances, the NSCs migrated slightly beyond the tumor edge n conjunction with— as if following or "trailing"—individual nfiltrating tumor cells (Fig. 2*C*, arrow). This phenomenon was most ıramatic when examined in the context of the more virulent and ıggressively infiltrative CNS-1 glioblastoma cell line (11) (Fig. 2*D*), vhich, in adult nude mice, demonstrates single cell invasive char-ıcteristics analogous to those of human glioblastomas. After im-ılantation as per Paradigm 1, extensive migration and distribution ıf β-gal$^+$ donor cells was again noted throughout the darkly ed-stained infiltrating tumor mass, up to, and along the encroach-ng tumor edge (red arrowheads), with further migration into the surrounding tissue in concert with and in virtual juxtaposition to ıggressively invading tumor cells (Fig. 2*D*, black arrows).

ISCs "Track" Infiltrating Tumor Cells. To better distinguish single umor cells migrating away from the main tumor mass, CNS-1 lioblastoma cells were labeled *ex vivo* by retroviral transduction of GFP cDNA before implantation (12). After implantation (as per Paradigm 1) of *lacZ*-expressing NSCs into the GFP-expressing CNS-1 tumor bed (Fig. 2 *E–L*), NSCs could not only be seen to distribute themselves throughout the tumor to its invading edge (Fig. 2 *E* and *F*), but could even more clearly be seen to "trail" islands of tumor cells migrating away from the main tumor mass (Fig. 2 *G* and *H*) as well as individual aggressive, dark red, or GFP$^+$, elongated infiltrating tumor cells (Fig. 2 *I–L*). Of note is the frequently observed apposition of transgene-expressing NSCs to invasive tumor cells (Fig. 2 *J–L*, arrows). The NSCs themselves never became tumorigenic. BrdUrd pulsing of animals before sacrifice confirmed prior observations that donor NSCs were quiescent in normal tissue (5), nonmitotic (i.e., BrdUrd$^-$) in the heart of the tumor, and with an occasional NSC that could still incorporate BrdUrd at the advancing edge, a situation optimal for targeting therapy toward invading tumors. The vast majority of NSCs remained not only quiescent but undifferentiated, expressing only nestin.

NSCs Implanted Intracranially at Distant Sites Migrate Toward Tumor. To determine whether NSCs have the capacity to migrate specifically toward the tumor, NSCs were injected into uninvolved intracranial sites distant from the main tumor mass in three separate paradigms (Fig. 3). In each case, donor NSCs migrating through normal adult tissue "targeted" the tumor. In Paradigm 2, NSCs were injected behind the glioblastoma. NSCs were always found distributed within the main tumor bed, as well as in apposition to migrating tumor cells in surrounding tissue (Fig. 3 *A* and *B*), with very few NSCs in other locations. In Paradigm 3, murine NSCs were injected into the contralateral hemisphere. NSCs (fluorescent red or X-Gal$^+$ blue) were seen migrating across the corpus

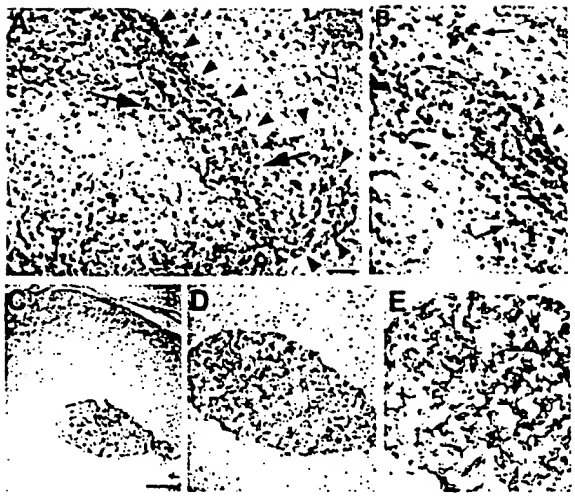

Fig. 5. Human NSCs (hNSCs) possess tumor tracking characteristics. (*A* and *B*) Rodent CNS-1 glioblastoma cells and human NSCs were implanted as per Paradigm 3 into opposite hemispheres of an adult mouse. Pictured 7 days later at low power (*A*) and high power (*B*) is a section through the neutral red-stained tumor (outlined by arrowheads) intermixed with human NSCs (identified by their brown nuclei following reaction with an anti-human nuclear antibody) (arrows) that migrated from the contralateral side. (*C–E*) Human HGL21 glioblastoma cells and hNSCs were similarly implanted into opposite hemispheres. Pictured at progressively higher power are sections through that neutral red-stained tumor intermixed with human NSCs (X-Gal$^+$ blue) that migrated from the contralateral side. (Scale bars: *A*, 20 μm, 15 μm in *B*; *C*, 60 μm, 30 μm in *D*, and 15 μm in *E*.)

callosum and central commissures (Fig. 3 *C–E*, arrows), toward the tumor (fluorescent green or dark neutral red$^+$ masses delineated by arrowheads in Fig. 3*F*), ultimately entering and populating (Fig. 3 *G* and *H*, arrows) the tumor on the opposite side of the brain. In Paradigm 4, NSCs were injected into the ipsilateral or contralateral cerebral ventricle. NSCs (blue) again were seen within the main tumor bed (Fig. 3*I*), as well as in juxtaposition to migrating "islands" of tumor cells (dark red) (Fig. 3*J*). The only source of blue cells in these paradigms was the "distant" NSC implant. Very few NSC-derived cells were found in normal brain tissue beyond the injection site, except when tracking toward the main tumor mass or near infiltrating tumor cells, suggesting that, whereas NSCs migrated freely within the tumor, the normal adult brain parenchyma presented a less permissive environment for migration. The NSCs themselves never became tumorigenic. The tumors in transplant recipients were never larger than those in non-transplant recipients. NSC-derived cells continued to express their *lacZ* transgene exuberantly, often in direct contact with tumor cells. These NSC behaviors appeared to be independent of the size or location of the tumor; findings were similar for large tumors, small tumor foci, and even single scattered tumor cells surrounding the main tumor mass. Fibroblasts grafted as controls never showed this dispersion or tropism, consistent with previous reports (15).

NSCs Injected into Systemic Circulation "Target" Intracerebral Gliomas. Murine NSCs were injected into the tail vein of adult nude mice in which a CNS-1 glioblastoma had been established 1 wk before in the frontal lobe. Four days after i.v. NSC injection, albeit with low efficiency, anti-β-gal$^+$ NSCs were distributed throughout the intracerebral tumor mass, but were not found in surrounding normal-appearing brain tissue, elsewhere in the brain, or in the brains of control animals (NSC-injected mice without intracerebral gliomas or tumor-bearing mice in the absence of NSC-injection).

Human NSCs and NSCs Expressing a Therapeutic Gene Migrate to Tumors. Because of the clinical implications of these migratory phenomena, we asked two further questions: (*i*) did these migratory

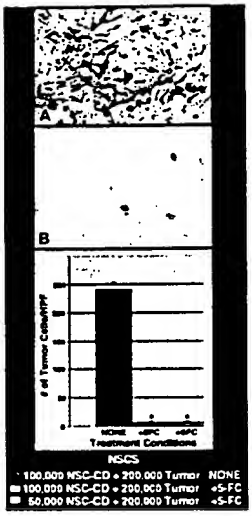

Fig. 6. Bioactive transgene (CD) remains functional (as assayed by *in vitro* oncolysis) when expressed within NSCs. CNS-1 glioblastoma cells (red) were cocultured with CD-transduced murine NSCs (*A* and *B*) (blue). Cocultures unexposed to 5-FC grew healthily and confluent (*A*), whereas plates exposed to 5-FC showed dramatic loss of tumor cells (*B*), represented quantitatively by the histograms (•, $P < 0.001$). The oncolytic effect was identical whether 1×10^5 CD-NSCs or half that number were cocultured with a constant number of tumor cells. (In this paradigm, subconfluent NSCs were still mitotic at the time of 5-FC exposure and thus also subject to self-elimination by the generated 5-fluorouracil and its toxic metabolites.)

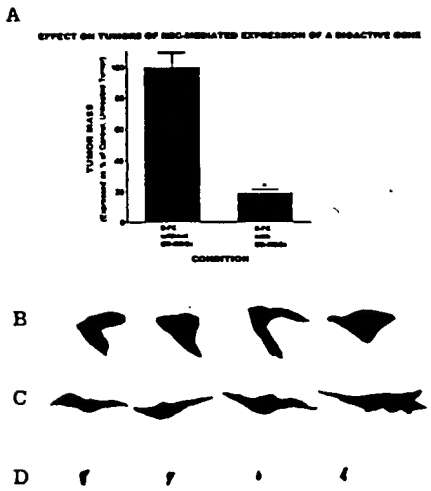

Fig. 7. Expression of a bioactive transgene (CD) delivered by NSCs is retained *in vivo* as assayed by reduction in tumor mass. The size of an intracranial glioblastoma populated with CD-NSCs in an adult nude mouse treated with 5-FC was compared with that of tumor treated with 5-FC but lacking CD-NSCs. These data, standardized against and expressed as a percentage of a control tumor populated with CD-NSCs receiving no treatment, are presented in the histograms in *A*. These measurements were derived from measuring the surface area of tumors (like those in Figs. 2–5), representative camera lucidas of which are presented in *B–D*. Note the large areas of a control non-5-FC-treated tumor containing CD-NSCs (*B*) and a control 5-FC-treated tumor lacking CD-NSCs (*C*) as compared with the dramatically smaller tumor areas of the 5-FC-treated animal who also received CD-NSCs (*D*) (≈80% reduction as per the histogram in *A*; •, $P < 0.001$), suggesting both activity and specificity of the transgene. The lack of effect of 5-FC on tumor mass when no CD-bearing NSCs were within the tumor (*C*) was identical to the effect of CD-NSCs in the tumor without the gene being used (*B*).

properties extend to human NSCs? and (*ii*) could relevant bioactive genes be expressed?

In answer to the first, human NSCs (brown nuclei, arrows in Fig. 5 *A* and *B*) transplanted into the hemisphere *contralateral* to a CNS-1 glioma (Paradigm 3) indeed migrated across the corpus callosum and infiltrated and distributed themselves throughout the targeted tumor (arrowheads) as previously observed. That human NSCs could similarly target a true human glioblastoma is suggested in Fig. 5 *C–E* in which Paradigm 3 was repeated employing human NSCs implanted contralateral to an HGL-21-derived tumor established in the nude mouse cerebrum; again, human NSCs migrated from one hemisphere to the other to infiltrate the glioblastoma.

To address the second question, NSCs were stably transduced with a transgene encoding the enzyme CD. CD can convert the nontoxic "prodrug" 5-FC to the oncolytic drug 5-fluorouracil, a chemotherapeutic agent that readily diffuses into tumor cells and has selective toxicity to rapidly dividing cells (18, 19). The CD gene provided an opportunity to examine a prototypical bioactive gene with a relevant, specific, quantifiable read-out of functionality (oncolysis) that might be enhanced by tumor proximity. CD-bearing NSCs retained their extensive migratory, tumor-tracking properties. To determine quantitatively whether a gene such as CD within an NSC retains its bioactivity—as assayed in this case by its anti-tumor effect—CD-bearing NSCs were first cocultured with glioma cells and then, when nearly confluent (Fig. 6*A*), exposed to 5-FC. Death of surrounding tumor cells was induced (Fig. 6*B*), even when the ratio of NSCs-to-tumor cells was as low as 1:4. NSCs that were mitotic at the time of 5-FC exposure self-eliminated. Control plates of tumor alone were not significantly killed by the same dose of 5-FC. To determine whether this bioactivity was retained *in vivo*, we used CD-transduced NSCs to express this gene within an intracranial glioma established in an adult nude mouse (3.5×10^4 NSCs to 7×10^4 CNS-1 tumor cells in a 1:2 ratio). After systemic treatment with 5-FC, there was dramatic (≈80%) reduction in the resultant tumor mass at 2 wk postimplantation as compared with that in untreated animals (Fig. 7), indicative of CD bioactivity.

Discussion

Transplanted NSCs have recently been recognized for their remarkable ability to migrate throughout the CNS, become normal constituents of the host cytoarchitecture, and disseminate bioactive molecules and retroviral vectors (3–10). Intriguingly, this migratory ability emulates the invasive spread of some brain tumors, e.g., gliomas. Here, we show that an unanticipated benefit of the directed, migratory capacity of transgene-expressing NSCs, including of human origin, may be to target invasive primary brain tumors (also including of human origin) that have proven refractory to current treatments (1, 2). Most gene therapy strategies employ viral vectors to deliver genes directly to tumor cells *in vivo*; however, the distribution of genes to the extensive regions and large numbers of cells in need of attack has been limited. The present study demonstrates the ability of NSCs to migrate expeditiously throughout a tumor mass and, presumably drawn by the degenerative or inflammatory environment created at the infiltrating tumor edge, to "surround" the invading tumor border, all while continuing to express a bioactively relevant transgene. Moreover, the foreign gene-expressing NSCs seem to follow or "track"—virtually ride "piggy-back" upon—those aggressively infiltrating tumor cells escaping into normal tissue. Although the NSCs migrate freely through the tumor, the normal adult brain parenchyma seems to present a less permissive environment for their migration, except when NSCs (even at sites distant from the tumor) travel in a directed fashion through normal adult CNS tissue to target the main tumor mass as well as individual infiltrating, tumor cells. The practical implication is that NSCs might actually "seek out" tumor foci that may have migrated undetected far away from the main tumor mass, not an uncommon occurrence with glioblastoma. Such behaviors are not displayed by cells of nonneural origin (15). Indeed, targeting may be so powerful that even NSCs injected into

the systemic circulation may preferentially populate intracerebral gliomas.

Hence, NSCs evince extensive tropism for the tumor itself or for the degenerating CNS it engenders. That the tumor itself elaborates at least some of the tropic cues is suggested by our *in vitro* studies in which glioma cells in culture, isolated from surrounding brain, prompted NSCs to migrate, by both contact and non-contact-mediated factors. Alternatively, or in addition, tropic cytokines may be released by extensively damaged normal tissue. That minor CNS destruction alone could not prompt the dramatic migration seen *in vivo* was suggested by NSC transplants into "mock" tumor bearing animals, i.e., animals in which a needle was inserted to emulate the tissue damage of establishing an experimental tumor bed but without the actual implantation of glioblastoma cells; NSCs did not migrate toward the site in this circumstance. Nevertheless, in other previously reported experimental situations in which significant neuronal death was rendered (13), NSC differentiation was altered by apparent trophic influences. Therefore, the signals to which the NSCs are responding are most likely complex, from multiple sources, and representing a "mixture" of attractants, adhesion and substrate molecules, chemokines, etc. Of broader biological significance, these findings suggest that migration can be unexpectedly extensive, even in adult brain and along nonstereotypical routes, if pathology (as modeled here by tumor) is present.

Having documented this powerful tropic interaction between NSCs and intracranial pathology, we believe that exogenous NSCs, genetically engineered *ex vivo* and strategically implanted, may provide a "platform" for the dissemination of therapeutic genes and/or gene products to previously inaccessible infiltrating tumor cells. As suggested in our CD/5-FC prodrug paradigm, NSCs were able to express a bioactive transgene *in vivo* to effect a significant biologically relevant read-out (dramatic reduction in tumor burden). Cytotoxic 5-fluorouracil and its toxic metabolites can readily diffuse into surrounding tumor cells giving CD an impressive "bystander" effect; as little as 2% of the tumor mass containing CD-expressing cells may generate significant oncolysis (22). Indeed, NSCs engineered to express CD are attractive as molecular pumps because they can generate agents that kill tumor cells yet undergo self-elimination should the NSCs themselves become mitotic. This prototypical genetic strategy represents one of many potential approaches to treating brain tumors with migratory genetically engineered NSCs. Other candidates include genes encoding: proteins that induce differentiation of neoplastic cells and/or their signal-transduction mediators; cell cycle modulators; apoptosis-promoting agents; anti-angiogenesis factors; immune-enhancing agents (23); fusion agents; and oncolytic factors (24). That these same engraftable migratory NSCs have been demonstrated to serve as intracerebral viral vector producer cells (10) may allow "extended" delivery of lethal viral-mediated genes to larger numbers of tumor cells in broader regions of brain. Instilled into the resection or biopsy cavity, or applied intermittently into or near the tumor mass or suspicious tissue or into cerebral ventricles, engineered NSCs could be used in conjunction with other interventions. An NSC-based strategy—responding to the altered biology of the abnormal adult brain and by virtue of their unique inherent biology—may both optimize present approaches and make feasible new ones for more effectively, selectively, and safely targeting genes and vectors to refractory, migratory, invasive brain tumors.

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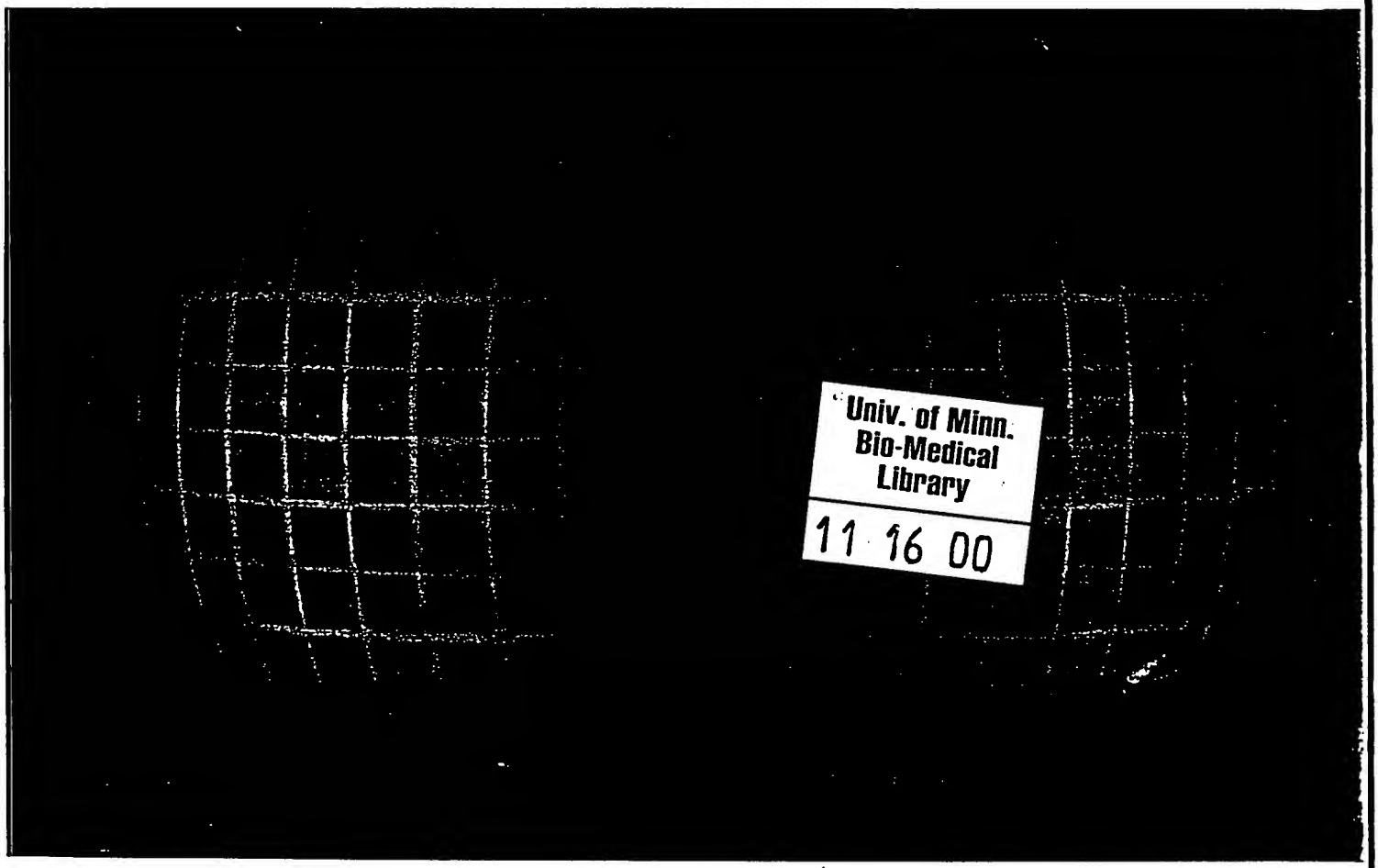

Novartis Foundation Symposium 231

NEURAL TRANSPLANTATION IN NEURODEGENERATIVE DISEASE: CURRENT STATUS AND NEW DIRECTIONS

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Neural stem cells are uniquely suited for cell replacement and gene therapy in the CNS

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Abstract. In recent years, it has become evident that the developing and even the adult mammalian CNS contain a population of undifferentiated, multipotent cell precursors, neural stem cells, the plastic properties of which might be of advantage for the design of more effective therapies for many neurological diseases. This article reviews the recent progress in establishing rodent and human clonal neural stem cell lines, their biological properties, and how these cells can be utilized to correct a variety of defects, with prospects for the near future to harness their behaviour for neural stem cell-based treatment of diseases in humans.

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Neurological disorders, whether hereditary or acquired, are typically characterized by a variety of cellular and molecular defects. The situation is aggravated by the fact that, during its maturation, the CNS appears progressively to lose its restorative capacity by establishing a potent inhibitory environment to neural regrowth and the formation of new connections, and by the formation of a blood–brain barrier (BBB) that protects the brain from blood-borne pathogens but also prevents the entrance of many therapeutic substances from the vascular compartment. Current attempts to promote CNS repair address these obstacles using the following strategies: (i) replacing affected cell populations (or structural components like

[1]This chapter was presented at the symposium by Professor Snyder, to whom correspondence should be addressed.

myelin) and their connections by neural grafts; (ii) providing trophic support by the introduction of neurotrophins and/or cytokines to diminish or prevent progressive neurodegeneration, stimulate neurite outgrowth, guide growing axons to their targets and promote establishment of functional synapses; and (iii) replacing missing neuroactive molecules, such as enzymes and neurotransmitters.

For several decades, fetal neural transplants have been used to promote CNS repair and formed the basis of an important branch of restorative neurobiology (reviewed in Dunnett & Björklund 1994, Fisher & Gage 1993, 1994). They have not only provided us with a wealth of information about normal CNS development but have generated invaluable information regarding the extent to which the perinatal, juvenile and adult CNS is able to react to growth signals and to mobilize dormant, intrinsic plastic capacities. In the majority of cases, fetal grafts have been used in three ways (Kordower & Tuszynski 1999, Marciano et al 1989): (i) to replace cellular elements, especially neurons, in the degenerating host parenchyma and to reinnervate targets which have lost their proper input; (ii) to act as tissue 'bridges' for host axonal regeneration due to their highly growth-permissive environment; and (iii) to prevent degeneration of host cells. More recently, an additional, and as yet hardly explored, phenomenon concerning graft/host interaction has been described, namely, the graft's potential to evoke robust restorative mechanisms within the juvenile recipient's brain which later result in an unusually well-remodelled cytoarchitecture originating almost exclusively from the host (Ourednik et al 1993, 1998, Ourednik & Ourednik 1994). Nevertheless, despite the fact that fetal grafts are already being used with likely success in human parkinsonian patients (Kordower et al 1995), a routine use of fetal tissue raises significant concerns, both biological and ethical, such as the availability of requisite amounts of suitable material and insuring survival of desired cells in a tissue that is typically heterogeneous. Moreover, with respect to the more and more popular idea of transferring therapeutic genes (or their end metabolic products) to the brain, primary fetal tissue is, due to its heterogeneity, not well suited for the genetic engineering (see below) that might be necessary to provide greater or more stable amounts of a trophic factor or to replace a particular enzyme in a defined cell type.

The transfer of a transgene or a gene product is frequently an important step in the attempt to correct a deficiency in the CNS. For this reason, many methods of gene transfer are under investigation (Breakfield et al 1999). They fall into two categories: mechanical delivery of DNA into cells *in vitro* and introduction of genetic material by virus-based vectors. Mechanical ways of introducing DNA into cells rely on diverse means of particulation and concentration of the DNA around the cell membrane in form of precipitates, liposomes, gold particles or molecular conjugates internalized by the cells in an active (endo- or pinocytosis) or passive (membrane fusion, electroporation or bombardment) process.

Although frequently employed, this methodology is not very efficient and cells may not get transfected in a stable manner.

Virus-based delivery of foreign genes into mammalian cells has several advantages: the small genome of retroviruses (still the most frequently used type of virus) allows relatively easy manipulation and insertion of larger transgene sequences, viruses can be grown to high titres in culture, infection efficiency is extremely high (close to 100% of cultured cells), and the DNA, in most cases, gets stably integrated into the genome in form of a provirus. However, care must be taken that the transgene is inserted into a replication-defective virus where all the transforming oncogene sequences have been removed. All the products necessary for replication and integration of such defective viruses are provided *in trans* by replication-competent but non-transforming helper viruses which later have to be completely removed from the purified vector — often a rather difficult task.

The introduction of DNA by viral vectors can be achieved either by *in situ* application, i.e., direct injection of genetically-altered viruses into the CNS, or by *ex vivo* gene therapy, where vector-mediated gene transfer into cells occurs *in vitro* and these transgenic cells are then transplanted into the brain regions of interest. Besides the technical difficulties inherent to the vectors used and common to both strategies (e.g. expression of viral genes, initiation of an antiviral immune response, reversion of the viral vector to a replication-competent state, and inactivation of transcription and/or expression of foreign genes), both suffer from specific insufficiencies as well. Thus, although progress is being made in targeting post-mitotic neural tissue with viral vectors like lentivirus, adenovirus (AV), adenoassociated virus (AAV), or herpes simplex virus (HSV) expressing therapeutic transgenes under cell type-specific promoters, they still may not address the widespread, extensive lesions characteristic of many neurodegenerative conditions, particularly those of genetic, perinatal, metabolic, inflammatory, infectious or traumatic origin. Furthermore, such strategies depend on relaying new genetic information through established endogenous neural populations and circuits, which, in fact, may have degenerated or failed to develop.

In the alternative approach, the *ex vivo* gene therapy strategy, the challenge comes in selecting a cellular population that can be easily altered genetically to produce a desired protein and then safely and efficiently introduced into discrete or widespread regions of the brain where they can reside innocuously and continue to deliver their genetic 'payload'. Donor cells may be chosen to act as miniature 'pumps' providing a source of exogenous substances that can diffuse to appropriate targets, to become integral members of the host cytoarchitecture and circuitry, or, ideally, to do both. Neurons would seem to be the appropriate cellular candidate for both delivering products to and integrating within the CNS. However, there are restrictions on the types and ages of neurons that survive grafting in a

functionally meaningful way for prolonged periods. Also, because mature neurons irreversibly stop proliferating, they are unsuitable targets for retrovirus-based gene delivery. While improvements in the design of integrating vectors (lentivirus, AAV) and episomal vectors (HSV, AV) may ultimately facilitate genetic manipulation of post-mitotic neurons *ex vivo*, at present, the usefulness of primary neurons as vehicles for gene transfer is minimal. Researchers therefore soon turned towards well-established cultures of non-neuronal cells which do proliferate and can easily be manipulated by retroviruses to express transgenes. Fibroblasts quickly became the ideal candidates and have been modified to produce a variety of neurotransmitter-synthesizing enzymes (e.g. tyrosine hydroxylase and choline acetyltransferase) and trophic proteins (e.g. nerve growth factor). Drawbacks of this technique are, however, that fibroblasts are unable to incorporate functionally into the host brain's cytoarchitecture, damaged circuits cannot be reformed and regulated release of substances may be missing. Thus, investigators started to look for another cell type which would be a source of a homogeneous cell population; which, while proliferating, could easily be maintained and genetically manipulated *in vitro*; and, at the same time, would, after grafting, be able to integrate seamlessly into the cytoarchitecture and circuitry of the host CNS.

For several years, there has been a growing interest in the therapeutic potential of neural stem cells (NSCs) and progenitors for therapy in CNS dysfunctions. This interest derives from the realization that these cells are more than simply a replacement for fetal tissue in transplantation paradigms and yet another vehicle for gene delivery. Rather, the basic biology of these cells endows them with a quality that other vehicles for gene therapy and repair may simply not possess (e.g. Martínez-Serrano & Snyder 1999, Snyder & Senut 1997): the potential to integrate into the neural circuitry after transplantation. With the first recognition that NSCs, propagated in culture, could be reimplanted into mammalian brain where they could reintegrate appropriately and stably express foreign genes, gene therapists and restorative neurobiologists began to speculate how such a phenomenon might be harnessed for therapeutic advantage. These, and the studies which they spawned (Yandava et al 1999, Lundberg et al 1997, Rosario et al 1997, Lacorazza et al 1996, Renfranz et al 1991) provided hope that the use of NSCs, by virtue of their inherent biology, might circumvent some of the limitations of presently available graft material and gene transfer vehicles, and make feasible a variety of novel therapeutic strategies (Table 1).

NSCs are postulated to be immature, uncommitted cells that exist in the developing and even adult nervous system (Gage et al 1995, Reynolds & Weiss 1992) and are responsible for giving rise to the vast array of more specialized cells of the mature CNS. They are operationally defined by their ability to self-renew, their potential to differentiate into various (if not all) neuronal and glial cell

TABLE 1 Properties of neural stem cells that make them appealing vehicles for CNS gene therapy and repair

Genetic manipulability
Progenitor/stem cells easily transduced *ex vivo* by most viral and non-viral gene transfer methods.
Facile engraftability following simple implantation procedures
From engraftment in germinal zones (as well as into parenchyma), can broach BBB unimpeded; no requirement for conditioning regimes (e.g. irradiation as in bone marrow transplantation or opening of BBB).
Sustained foreign (therapeutic) gene expression
Throughout CNS, from fetus to adult, following technically simple and safe reimplantation procedures; CNS levels rise immediately.
Potential for normal reintegration into host cytoarchitecture and circuitry
Differentiate along all CNS cell-type lineages; important for diseases in which neurons and glia are both affected; not only allows direct, stable and perhaps regulated delivery of therapeutic molecules, but also enables replacement of range of dysfunctional neural cells and possible reconstruction of connections and networks.
Ability to migrate
Particularly within germinal zones, enabling replacement of genes and cells to be directed not only to discrete sites but to widely disseminated lesions as well for diseases of a more global nature; for more focal implants, ability of cells to intermingle with host cells rather than clump at injection track insures homogeneous distribution of therapeutic molecules throughout target tissue.
Plasticity
Ability to accommodate to region of engraftment and assume array of phenotypes; obviates necessity for obtaining donor cells from many specific CNS regions, or imperative for precise targeting of donor cells during reimplantation, or need for tissue-specific promoters for foreign gene expression.
Compensatory of transgene non-expression
Low levels of normal neural products expressed intrinsically by progenitor/stem cells (lysosomal enzymes; neurotrophic, matrix, adhesion and homeodomain molecules; myelin) helps safeguard against transgene inactivation; neural cells may sustain expression of foreign neural genes longer than non-neural vehicles; ability to integrate multiple copies of a transgene into its genome (e.g. following repeated sequential retroviral infection) helps thwart loss of expression; may also provide as-yet-unrecognized beneficial neural-specific substances.
One stem cell may carry multiple transgenes
Following multiple transfection events, one cell can transfer multiple gene products simultaneously.
Minimization of side effects
Distribution of gene products restricted to CNS; while proteins may be disseminated by stem cells throughout brain for diseases of global nature, by altering mode of administration, cells can be selectively integrated in proximity to neurons that require given factor without affecting cells for which the molecule might be problematic; conditioning regimes not required prior to transplantation as in bone marrow therapy.

(*Continued*)

TABLE 1 (*Continued*)

Ability to serve as producer cells for the in vivo dissemination of viral vectors
May help amplify distribution of virus-mediated genes to large CNS regions and numbers of cells.
Immunotolerance
In rodent transplant studies, multiple recipients and mouse strains can integrate the same murine stem cell clone without rejection or the necessity for immunosuppression, suggesting a need for generating very few effective clones (one clone used by many).
Tropism for and trophism within regions of CNS degeneration
When confronted with neurodegenerative environments, stem cells alter their migration & differentiation patterns towards replacement of dying cells; probably a vestigial developmental strategy with therapeutic value.

lineages, and to populate developing or degenerating CNS regions in multiple regional and temporal contexts. We can even hypothesize that, whenever the CNS is injured, it may actually try to 'repair itself' with its own endogenous NSC population but that, for most injuries that come to clinical attention, that supply is restricted in the number of available NSCs or insufficiently mobilized and even counteracted by growth-inhibitory environment, specially in adult brain. This unexplored possibility of a 'self-repair' could already be postulated in the context of the regenerative effect of fetal tissue grafts on host tissue (Ourednik et al 1993, 1998, Ourednik & Ourednik 1994) and is corroborated by the fact that such fetal tissues still contain a large pool of endogenous NSCs which are probably responsible for the observed graft-induced remodelling by the post-mitotic host brain. Furthermore, pilot studies in which endogenous progenitors in the suventricular germinal zone (SCZ) are labelled and tracked just as a devastating hypoxic–ischaemic brain injury is experimentally imposed on the cortex, suggests that these progenitors alter their normal stereotypical migratory route to the olfactory bulb and move instead towards the damaged regions to become new neurons in regions where neurogenesis has been conventionally deemed as having been completed. Therefore, to augment such a response with 'pure' exogenous NSCs (transgenic or not), implanted opportunistically at strategic times following injury, may enable an even more significant recovery.

NSC clones have been maintained in a proliferative state by several equally safe and effective strategies: through manipulation of 'internal commands' by genetic means (e.g. transduction of propagating genes that interact with cell cycle regulatory proteins) or by exposure to 'external commands' (e.g. such epigenetic means as chronic mitogen stimulation or co-culture on various cellular membrane substrates). Such manipulations do not subvert the ability of stem cells to respond to normal microenvironmental cues: to withdraw from the cell cycle, interact with

host cells and to differentiate. This point has been successfully illustrated by a prototypical model murine NSC clone (designated clone C17.2), which was initially isolated from 4-day-old mouse cerebellum but has the ability to accommodate to most neural regions at most periods throughout the mouse's life (Snyder et al 1992). When transplanted into various germinal zones throughout the brain, these cells participate in normal development of multiple regions at multiple stages along the murine neuraxis (expressing their marker, β-galactosidase, from the bacterial *lacZ* transgene). They intermingle non-disruptively with endogenous neural progenitor/stem cells, responding to the same spatial and temporal cues in a similar manner and differentiating into all neuronal and glial cell types. Crucial for therapeutic considerations, the structures to which they contribute develop normally. Thus, their use as graft material can be considered almost analogous to haematopoietic stem cell-mediated reconstitution. In the following sections, using clone C17.2 as model for NSCs in general, we present examples of their behaviour in several grafting experiments simulating various neuropathological situations.

Non-engineered NSCs correct a variety of CNS defects

In testing the potential of NSCs to replace dying cells and lost neural circuits in degenerating brain, insights have been derived from studying mouse mutant and specific injury paradigms which have served nicely as well-controlled and well-defined models for more complex CNS dysfunctions. In such experiments, NSCs appear well suited for replacing some degenerated or dysfunctional neural cells.

In the *meander tail* (*mea*) mutant, which is characterized by failure of sufficient granule neurons to develop in certain regions of the cerebellum, NSCs, implanted at birth, were capable of 'repopulating' large agranular portions with neurons (Rosario et al 1997). A pivotal observation, with implications for fundamental stem cell biology, was that cells with the potential for multiple fates 'shifted' their mode of differentiation to compensate for a deficiency in a particular cell type. As compared with their differentiation in normal cerebella, a preponderance of these donor NSCs in regions deficient in granule neurons pursued a granule neuronal phenotype in preference to other potential phenotypes, suggesting the presence of environmental signals 'pushing' undifferentiated, multipotent cells towards repletion of the inadequately developed cell type. This phenomenon was observed in more than one study (as will be illustrated in some of the following examples) and presents a possible developmental mechanism with obvious therapeutic value.

Preliminary work in another mutant, the *reeler* (*rl*) mouse, has suggested that NSCs may not only replace developmentally impaired cells, but may also help correct certain aspects of abnormal cytoarchitecture (Auguste et al 1996). The laminar assignment of neurons in *rl* mouse brain is profoundly abnormal due,

most likely, to a mutation in a gene encoding the secreted extracellular matrix (ECM) molecule, Reelin. NSCs, implanted at birth into the defective developing *rl* cerebellum, appeared in pilot studies not only to replace missing granule neurons in correct laminar position, but also to restore a more wild-type laminated appearance in engrafted regions by influencing the migration and survival of mutant neurons, most likely by providing molecules (including Reelin) that guide proper histogenesis. These findings therefore suggest a possible stem cell-based strategy for the treatment of CNS diseases characterized by abnormal cellular migration, lamination and cytoarchitectural arrangement.

Many neurologic diseases, particularly those of neurogenetic aetiology, are characterized by global degeneration or dysfunction. Mutants characterized by CNS-wide white matter disease provide an ideal model for testing hypotheses that NSCs might also be useful in neuropathologies requiring widespread neural cell replacement. The oligodendroglia of the dysmyelinated *shiverer* (*shi*) mouse are dysfunctional because they lack myelin basic protein (MBP) essential for effective myelination. Therapy, therefore, requires widespread replacement with MBP-expressing oligodendrocytes. NSCs transplanted at birth (employing an intracerebroventricular implantation technique devised for diffuse engraftment of enzyme-expressing NSCs to treat global metabolic lesions, Yandava et al 1999) resulted in engraftment throughout the *shi* brain with repletion of significant amounts of MBP (Figs 1 and 2). Accordingly, of the many donor cells which differentiated into oligodendroglia, a subgroup myelinated 40% of host neuronal processes. In some recipient animals, the symptomatic tremor decreased. Therefore, 'global' cell replacement seems feasible for some pathologies if cells with stem-like features are employed. This approach is being extended to other poorly myelinated mutants, e.g. mouse models of Krabbe's globoid cell leukodystrophy. The ability of NSCs to remyelinate is of particular importance because dys-/de-myelination plays an important role in many genetic (e.g. leukodystrophies, inborn metabolic errors) and acquired (traumatic, infectious, asphyxial, ischemic, inflammatory) neurodegenerative processes. More broadly, complementation studies in mutants, such as those described above, help support a NSC-based approach, whether with exogenous NSCs or with appropriately mobilized endogenous NSCs, for compensating for neurodevelopmental problems of many actiologies.

One of the most fascinating characteristics of NSCs is that they indeed can react to neurodegeneration by 'shifting' their pattern of differentiation towards 'replenishing' of the missing cell types. One of the studies demonstrating this phenomenon was performed in a model of experimentally induced apoptosis of selectively targeted pyramidal neurons in the adult mammalian neocortex (Snyder et al 1997). Apoptosis (at least at particular critical phases) is becoming implicated in a growing number of both neurodegenerative and normal

developmental processes. When transplanted into this neuron-specific degenerative environment, 15% of NSCs 'altered' the differentiation path they otherwise would have taken under normal developmental circumstances (neurogenesis has normally ceased in the adult cortex) and instead differentiated specifically into that type of degenerating neuron, partially replacing that lost neuronal population. Pilot studies further suggest that some replacement neurons sent axons across the corpus callosum to appropriate targets in the contralateral hemisphere. Thus, this neurodegeneration may have created a 'milieu' which recapitulates normal embryonic developmental cues (e.g. for cortical neuronogenesis) to which NSCs can respond to therapeutic advantage.

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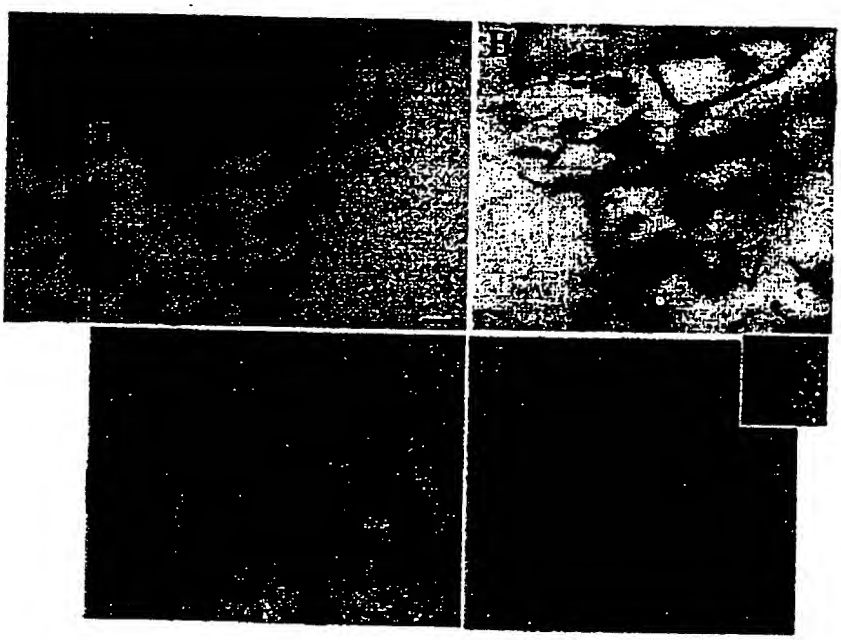

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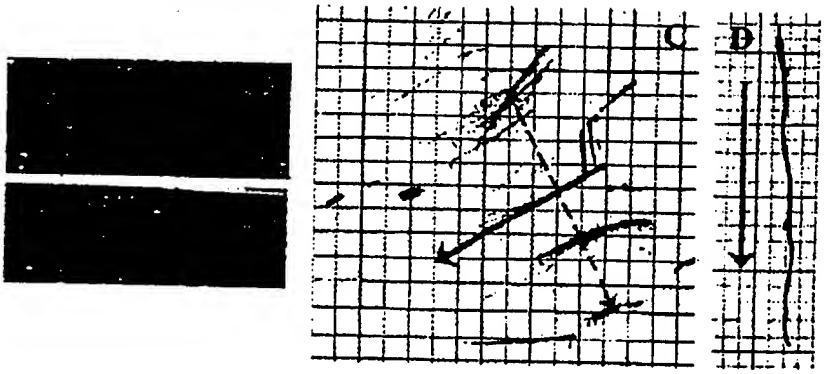

Evidence from experimental mouse models that even more closely emulate clinical situations further suggests that CNS injury or degeneration (of a certain type and/or during critical developmental time windows) might advantageously direct the migration, proliferation and differentiation of NSCs, both of host and donor origin. In a preliminary study, mice treated systemically with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a drug selectively destroying dopaminergic cells in the brainstem, and subsequently grafted unilaterally with NSC clone C17.2 displayed a reconstituted dopaminergic cell population

FIG. 1. Engrafted NSCs in recipient *shi* mutants differentiate into oligodendrocytes (I) and functional and behavioural assessment of transplanted *shi* mutants and controls (II). (I A,B) Donor-derived $Xgal^+$ cells in representative sections through the corpus callosum possess characteristic oligodendroglial features — small, round or polygonal cell bodies with multiple fine processes oriented in the direction of the neural fibre tracts. (C) Close-up of a representative donor-derived anti-β-galactosidase immunoreactive oligodendrocyte (arrow) extending multiple processes toward and beginning to enwrap large, adjacent axonal bundles viewed on end in a section through the corpus callosum. That cells such as those in A–C are oligodendroglia is confirmed by the representative electron micrograph in (D), demonstrating a donor-derived Xgal-labelled oligodendrocyte (LO) distinguished by the electron-dense Xgal precipitate that typically is localized to the nuclear membrane (arrow), endoplasmic reticulum (arrowhead), and other cytoplasmic organelles. The area indicated by the arrowhead is magnified in the inset to demonstrate the unique crystalline nature of individual precipitate particles. (II) The *shi* mutation is characterized by the onset of tremor and a 'shivering gait' by the second to third postnatal week. The degree of motor dysfunction in animals was gauged in two ways: (i) by blindly scoring periods of standardized videotaped cage behaviour of experimental and control animals and (ii) by measuring the amplitude of tail displacement from the body's rostral–caudal axis (an objective, quantifiable index of tremor). Video freeze-frames of representative unengrafted and successfully engrafted *shi* mice are seen in (A) and (B). The whole-body tremor and ataxic movement observed in the unengrafted symptomatic animal (A) causes the frame to blur, a contrast to the well-focused frame of the asymptomatic transplanted *shi* mouse (B). 60% of transplanted mutants evinced nearly normal-appearing behaviour and attained scores that were not significantly different from normal controls. (C) and (D) depict the manner in which whole-body tremor was mirrored by the amplitude of tail displacement (hatched arrow in C), measured perpendicularly from a line drawn in the direction of the animal's movement (solid arrow, which represents the body's long axis). Measurements were made by permitting a mouse, whose tail had been dipped in India ink, to move freely in a straight line on a sheet of graph paper as shown. Large degrees of tremor cause the tail to make widely divergent ink marks away from the midline, representing the body's axis (C). Absence of tremor allows the tail to make long, straight, uninterrupted ink lines on the paper congruent with the body's axis (D). The distance between points of maximal tail displacement from the axis was measured and averaged for transplanted and untransplanted *shi* mutants and for unaffected controls (hatched arrow). (C) shows data from a poorly engrafted mutant that did not improve with respect to tremor whereas (D) reveals lack of tail displacement in a successfully engrafted, now asymptomatic *shi* mutant. Overall, 64% of transplanted *shi* mice examined displayed at least a 50% decrement in the degree of tremor or 'shiver'. Several showed zero displacement. Bars: I. A,B, and C = 10 μm, D = 1.5 μm, II. A,B = 2.5 cm. (Modified from Yandava et al 1999.)

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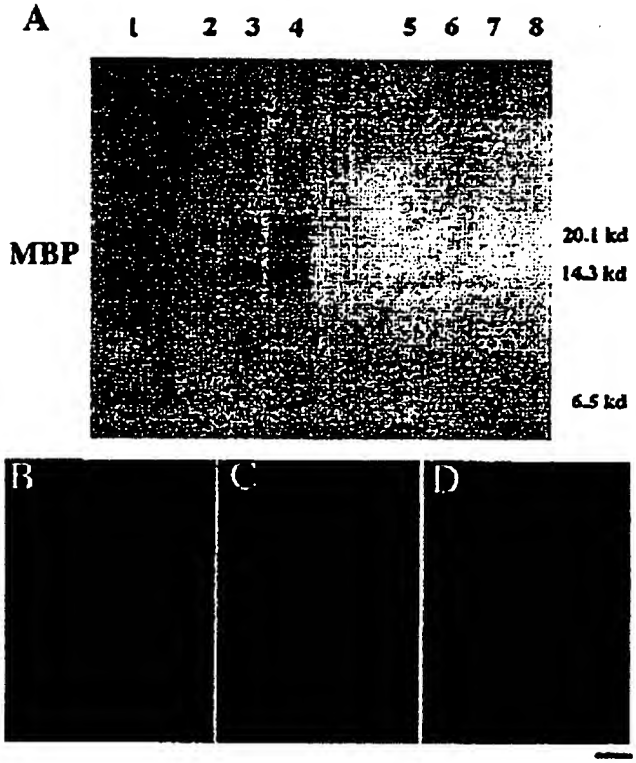

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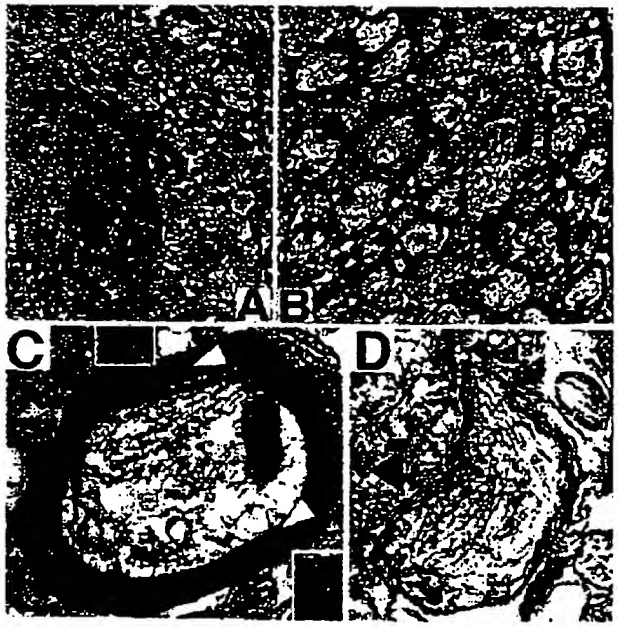
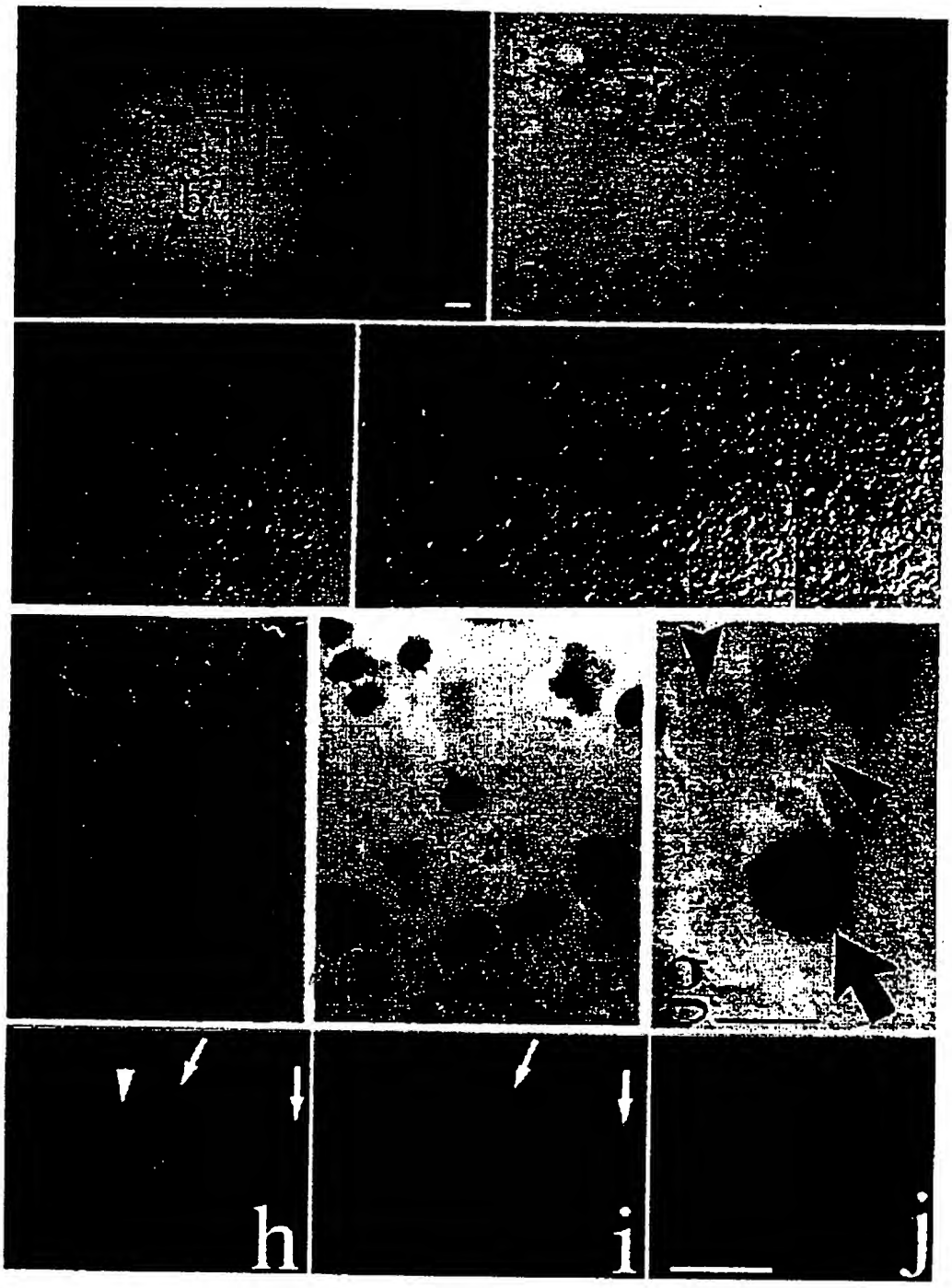


composed of donor and host. This suggests that NSCs not only replenished a defined pool of a missing cell type but also reactivated regenerative capacities within the aged host.

In a newborn mouse, unilateral carotid ligation combined with reduced ambient oxygen produces extensive hypoxic–ischaemic brain injury (HI) throughout the ipsilateral cerebral hemisphere. HI is an ideal prototype for a range of untreatable acquired and inherited neurodegenerative conditions and involves multiple cell types and regions in a devastatingly extensive manner. NSC clone C17.2 was used in two ways to help study the biology of NSC-based reconstitution of this large CNS lesion (Park et al 1997, 1999). In the first paradigm, NSCs are allowed to integrate during development into the representative cytoarchitecture of the normal brain prior to unilateral HI, creating virtually a chimeric brain of host and donor 'reporter stem cells'; the movements and responses of this 'reporter' NSC clone to HI (which can be reliably tracked by virtue of its *lacZ* reporter gene expression) would presumably mirror the behaviour of endogenous host progenitors and NSCs, with which it has intermixed, whose clonal relationships,

FIG. 2. (I) Myelin basic protein (MBP) expression in mature transplanted and control brains. (A) Western analysis for MBP in whole brain lysates. The brains of three representative transplanted *shi* mutants (lanes 2–4) express MBP at levels close to that of an age-matched unaffected mouse (lane 1, positive control), and significantly greater than the amounts seen in untransplanted (lanes 7,8, negative control) or unengrafted (lanes 5,6, negative control) age-matched *shi* mutants. (Identical total protein amounts were loaded in each lane.) (B–D) Immunocytochemical analysis for MBP. (B) The brain of a mature unaffected mouse is immunoreactive to an antibody to MBP (revealed with a Texas red-conjugated secondary antibody). (C,D) Age-matched engrafted brains from *shi* mice similarly show immunoreactivity. Untransplanted *shi* brains lack MBP. Therefore, MBP immunoreactivity has also classically been a marker for normal donor-derived oligodendrocytes (C,D). II. NSC-derived 'replacement' oligodendrocytes appear functional as demonstrated by ultrastructural evidence of myelination of *shi* axons. In regions of MBP-expressing NSC engraftment, *shi* neuronal processes become enwrapped by thick, better compacted myelin. (A) At 2 weeks post-transplant, a representative donor-derived, labelled oligodendrocyte (LO) (recognized by extensive Xgal precipitate (p) in the nuclear membrane, cytoplasmic organelles, and processes) is extending processes (a representative one is delineated by arrowheads) to host neurites, and is beginning to ensheathe them with myelin (m). (B) If engrafted *shi* regions, such as that in A, are followed over time (e.g. to 4 weeks of age as pictured here), the myelin begins to appear healthier, thicker and better compacted (examples indicated by arrows) than that in age-matched untransplanted control mutants. (C) By 6 weeks post-transplant, it matures into even thicker wraps; ~40% of host axons are ensheathed by myelin. The higher power view of a representative axon shows its myelin to be dramatically thicker and better compacted than the *shi* myelin (an example of which is shown in D) (black arrowhead) from an unengrafted region of an otherwise successfully engrafted *shi* brain. In C, white arrowheads indicate representative regions of myelin that are magnified in the adjacent insets; major dense lines are evident. (Modified from Yandava et al 1999.)



characteristics and degree of homogeneity are much less certain and which are otherwise 'invisible' to such monitoring. In the second paradigm, C17.2 'reporter cells' are implanted at various intervals following HI. In both paradigms, in response to HI, a subpopulation of 'reporter' and host cells transiently re-entered the cell cycle and migrated preferentially to the ischaemic site as if responding to newly elaborated cues. Donor-derived cells integrated extensively within the large infarcted areas that span the length of the brain; even cells implanted into the intact contralateral hemisphere migrated towards regions of injury. A subpopulation of both reporter- and host-derived cells (particularly in the penumbra) then once again became quiescent and differentiated into new neurons and oligodendrocytes, the neural cell types typically damaged following HI and least likely to regenerate spontaneously in postnatal CNS. In the injured postnatal neocortex there was a fivefold increase in donor-derived oligodendrocytes compared to the intact neocortex and, most significantly, NSCs now yielded neurons at a stage in mammalian development when no cortical neurons are normally born; 5% of engrafted NSCs on the injured side, compared to 0% on the intact contralateral side, now differentiated into neurons, an amount that translates into tens-of-thousands of replacement neurons. (While it is unknown how many neurons and how much circuitry are required to reconstruct a damaged system, older lesion data suggest that relatively little, even less than 10%, restoration may be sufficient.) As in the targeted apoptosis model, novel signals appear to be transiently elaborated following HI (three to seven days following HI appears

FIG. 3. Neuronal replacement by a representative human neural stem cell (hNSC) clone following transplantation at birth into the cerebellum of the granule neuron-depleted *meander tail* mutant mouse model of developmental neurodegeneration. (a–g) BrdU-intercalated donor-derived cells identified three weeks following direct implantation into external germinal layer of the *meander tail* (*mea*) cerebellum by anti-BrdU immunochemistry. (a) hNSCs are present in the inner granular layer (igl, arrows) of all lobes of the cerebellum (granule neurons are diminished *throughout* the cerebellum with some prominence in the anterior lobe). (b) Higher magnification of the representative posterior cerebellar lobe indicated by arrow in panel a, demonstrating the large number of donor-derived cells present within the recipient igl. (c–g) Various magnifications of donor-derived cells within the igl of a *mea* anterior cerebellar lobe. (f,g) Nomarski optics are utilized to bring out the similarity in site and morphology of host BrdU-negative cerebellar granule neurons (arrow heads) and a BrdU-intercalated, donor-derived neuron (arrow). (h, i) Neuronal differentiation of a subpopulation of donor-derived, BrdU-intercalated cells is illustrated by co-labelling with BrdU in (h) and the mature neuronal marker NeuN in (i) (indicated with arrows). Adjacent, donor-derived cells are non-neuronal as indicated by their BrdU-positive, NeuN-negative phenotype (arrowhead). (j) Cells within the igl are demonstrated to be donor-derived human cells by FISH for a human-specific probe identifying the centromeres of all chromosomes, *all*. Other centromeres are present, but out of the plane of focus in this photomicrograph. Bars: a,b = 100 μm; c, d = 75 μm; e = 40 μm; f,g = 10 μm; h,i,j = 50 μm. (Modified from Flax et al 1998.)

optimal), to which NSCs (donor and host) respond by 'shifting' their normal fate to compensate for the loss of those particular cell types.

These phenomena are probably pervasive throughout the CNS. A similar tropism and trophism for NSCs by apoptotic neurodegenerative environments appears evident in the postnatal spinal cord (SC) during segmental motoneuron (MN) degeneration induced by neonatal sciatic axotomy, a classic experimental model of spinal neuron degeneration. Although MNs are normally born only in the fetus, in pilot studies where NSCs are implanted during active degeneration, a significant proportion of them will engraft, migrate toward and throughout the segments of MN-impoverished ventral horn and differentiate (20%) into cells that resemble the lost MNs (Himes et al 1995). Again, engrafted NSCs continue to express foreign reporter genes suggesting that, as in the asphyxiated brain, implantation of genetically engineered NSCs expressing trophic agents, cytokines or other factors might enhance neuronal differentiation, neurite outgrowth and proper connectivity.

NSCs and genetic engineering — combining cell replacement and gene therapy

Precisely what the stem cell-modifying signals are that are normally elaborated as a consequence of neurodegeneration is an area of active investigation. They no doubt are a complex mix of various mitogens, cytokines, trophic and tropic agents, adhesion and ECM molecules, chemotactic and angiogenic factors, etc., elaborated by reactive astrocytes, activated microglia, inflammatory cells, invading macrophages and damaged neurons and glia. Since the concentrations of these factors and their ratios change with time, they create a temporal 'window' of increased plasticity during the acute/subacute post-lesioning phase (first 2–3 weeks). Might this naturally established plastic phase in the injured brain, so favourable for successful engraftment of NSCs, be further prolonged and enhanced by augmenting some of those factors? Because in the paradigms described above, engrafted NSCs continue to express their marker transgene *lacZ* within the large infarcted areas, it appears feasible that such cells can be genetically manipulated prior to transplantation to express such agents, *in vivo* (much like a pump infusing growth factors).

Neurotrophin 3 (NT-3) is known to play a role in promoting neuronal differentiation (although its presence after HI remains unclear). An NSC clone secreting large amounts of NT-3 might not only have an impact on host cells, but, intriguingly, might itself respond to NT-3 in an autocrine/paracrine fashion. This, indeed, seems to be feasible. In preliminary studies, when a subclone of C17.2 NSCs, retrovirally transduced *ex vivo* to overexpress NT-3, are implanted into brains suffering from HI, the percentage of donor-derived neurons seems to be

dramatically increased to 20% in the infarction cavity and to 80% in the penumbra (compared to the 5% when non-engineered NSCs are used) (Park et al 1997). As another example, we can again adduce the pilot experiments on parkinsonian mice we described above. A group of these mice were grafted with C17.2 cells overexpressing the neural cell adhesion molecule L1 (known to promote cell migration and neurite outgrowth; Burden-Gulley et al 1997). This resulted in an increased migration of donor-derived dopaminergic nerve cells and a quicker bilateral reorganization of the substantia nigra. Such observations suggest the use of NSCs for simultaneous, combined gene therapy and cell replacement in the same transplant using the same clone in the same recipient—an appealing NSC property with implications for therapies in other degenerative conditions involving other neural cell types.

The feasibility of a stem cell-mediated delivery system for therapeutic molecules was first affirmed by correcting the widespread neuropathology of a murine model of the genetic neurodegenerative lysosomal storage disease mucopolysaccharidosis type VII (MPS VII). Caused by a frameshift deletion of the β-glucuronidase gene (GUSB), this heritable condition causes progressive mental retardation in humans and inexorable neurodegeneration in mice (Snyder et al 1995). NSCs were genetically modified with a retrovirus encoding human GUSB to augment the mouse GUSB constitutively secreted by these cells. Transplantation of these GUSB-overexpressing cells into the cerebroventricular system of newborn MPS VII mice resulted in profuse incorporation of donor-derived cells throughout the mutant neuraxis. This brain-wide distribution of engrafted GUSB-secreting NSCs corresponded to the distribution of corrective levels of GUSB throughout the mutant brains devoid of that enzyme. The diffuse GUSB expression in turn resulted in widespread permanent correction of lysosomal storage in mutant neurons and glia, throughout mutant brains. While MPS VII may be regarded as 'uncommon', the broad category of diseases which it models (neurogenetic degenerative conditions) afflicts as many as 1 in 1500 persons. This approach is therefore being extended to other untreatable neurodegenerative diseases characterized by an absence of discrete gene products and/or the accumulation of toxic metabolites. For example, retrovirally-transduced NSCs, implanted into fetal and neonatal mice using the intracerebroventricular technique, have successfully mediated widespread expression throughout the brain of the α-subunit of β-hexosaminidase, a mutation of which leads to accumulation of GM2 ganglioside (Tay–Sachs disease, Lacorazza et al 1996).

With the stage having been set by experiments such as these, such *ex vivo* gene therapy strategies have been successfully employed in other experimental models of neurologic disease. These have included delivering tyrosine hydroxylase (TH) to the striatum of parkinsonian animals; nerve growth factor (NGF) to cholinergic systems of the septum and nucleus basalis magnocellularis to induce sprouting and

to reverse cognitive deficits in models of Alzheimer's disease and ageing; NGF and brain-derived growth factor (BDNF) for neuroprotection against excitotoxic lesions in striatum mimicking Huntington's disease (Martínez-Serrano & Björklund 1996, Martínez-Serrano et al 1995a,b, 1996). In approaching metabolic diseases such as those above, there is an interesting point to be made that might be applicable to *ex vivo* gene therapy in general: NSCs, because they are normal CNS cells, often constitutively express baseline amounts of a particular enzyme or neuroactive factor. The extent to which this amount needs to be augmented by genetic engineering may vary from protein to protein and needs to be studied individually. Reassuringly, in most inherited metabolic diseases and in many neurologic diseases in general, the amount of enzyme required to restore normal metabolism and forestall CNS disease may be quite small. Also reassuringly, we affirmed that NSC expression of therapeutic levels of foreign genes (even if reduced) can persist lifelong and that transducing a given NSC multiple times with a retroviral vector, thus inserting multiple copies of a therapeutic transgene within the same cell, is a simple and immediately available method for blunting decrements in transgene expression.

Experiments like those described above have established a paradigm for the stem cell-mediated brain-wide distribution of other diffusible (e.g. synthetic enzyme, neurotrophin, viral vector) and non-diffusible (e.g. myelin, extracellular matrix) therapeutic or developmental factors, as well as the distribution of 'replacement' neural cells. In developing brains, the cells actually contribute to organogenesis of multiple CNS structures (Table 1). Because NSCs can populate widely disseminated developing or degenerating CNS regions with cells of multiple lineages, their use as graft material in the brain can be considered analogous to haematopoietic stem cell-mediated reconstitution and gene transfer in the body. Yet, unlike in bone marrow transplantation, this method of delivery does not preclude being able to transport gene products into the cytoarchitecture of circumscribed regions in order to effect selective manipulations and avoid extensive genetic alteration should the clinical context demand it. For some diseases, widespread gene product dissemination is not desired. In fact, solely by altering their mode of administration or implantation, NSCs can be selectively integrated into more focal and discrete regions in proximity to those neurons that require a given neuroactive factor without affecting cells at more remote locations for which the molecule might be problematic.

A recent unexpected use of the NSC takes advantage of its ability to migrate extensively and to 'home in' selectively on CNS pathology while continuing to express bioactive foreign genes. One of the impediments to the treatment of primary human brain tumours (e.g. gliomas) has been the degree to which they expand, infiltrate surrounding tissue and migrate widely into normal brain, usually rendering them 'elusive' to effective resection, irradiation, chemotherapy

or gene therapy. In preliminary studies, we have observed that migratory NSCs, when implanted into experimental intracranial gliomas *in vivo* in adult rodents, distribute themselves quickly and extensively throughout the tumour bed and migrate uniquely in juxtaposition to widely expanding and aggressively advancing tumour cells while continuing to stably express a foreign gene. The NSCs, in effect, 'surround' the invading tumour border while 'chasing down' infiltrating tumour cells. Furthermore, when implanted intracranially at *distant* sites from the tumour bed in adult rodent brain (e.g. into normal tissue, into the contralateral hemisphere, or into the cerebral ventricles), the donor cells migrate through normal tissue targeting the tumour cells (including human glioblastomas). NSCs can deliver a bioactive therapeutically-relevant molecule — the oncolysis-promoting enzyme cytosine deaminase — such that *in vitro* and *in vivo*, upon activation, a dramatic, quantifiable reduction in surrounding tumour cell burden results. These data suggest the adjunctive use of inherently migratory NSCs as a delivery vehicle for more effectively targeting a wide variety of therapeutic genes and vectors to refractory, migratory, invasive brain tumour cells. More broadly, they suggest that NSC migration can be extensive, even in the adult brain and along non-stereotypical routes, if pathology (as modelled here by tumour) is present.

Getting closer to human therapy — establishment of human NSC clones

The ability of NSCs to migrate and integrate throughout the brain as well as to disseminate a foreign gene product is of great significance for the development of new therapies for neurodegenerative diseases in humans. Hereditary diseases like Tay–Sachs disease result in lesions throughout the CNS. Diseases of adult onset, too, e.g. Alzheimer's disease, can be diffuse in their pathology. Even acquired diseases such as spinal cord injury (SCI) are more extensive in their involvement than is typically assumed. Such trauma-related abnormalities may fully benefit from the multifaceted approach NSCs may enable: e.g. cell replacement to provide new neural connections as well as remyelination; gene therapy to support the survival of damaged neurons, to neutralize hostile milieu, to counteract a growth-inhibitory environment and promote neurite regrowth, and the re-formation of stable and functional contacts. In the case of chronic SCI, an improved knowledge of the molecular barriers to SC remodelling is needed to optimize the plastic behaviour of NSCs in this otherwise foreboding terrain.

A better understanding of fundamental NSC biology may soon allow human NSCs to be transplanted with therapeutic efficacy and without concern for recipient safety. Progress in this regard is already being made. Several NSC clones have been established from the human fetal telencephalon (hNSCs) which seem to emulate many of the appealing properties of their rodent counterparts

(Flax et al 1998): they differentiate, *in vitro* and *in vivo*, into neurons, astrocytes and oligodendrocytes; they follow appropriate developmental programmes and migrational pathways similar to endogenous precursors following engraftment into developing mouse brain; they express foreign genes *in vivo* in a widely disseminated manner; and they can replace missing neural cell types when grafted into various mutant mice (Fig. 3).

Although very promising for ultimate human CNS therapy, these findings need first to be reproduced in animals that are closer to humans. As the analyses from our first studies indicate, hNSCs seem to respond to patterning signals from the developing monkey brain and intermingle with the endogenous cell populations following established migration streams (Ourednik et al 1999). If hNSCs behave in lesioned primate brains with respect to engraftment and foreign gene expression as they seem to do in mice, we soon might have a powerful and versatile therapeutic tool in hand for use in human trials to help address genuine clinical neurodegenerative diseases — the ultimate goal of experiments that started almost a decade ago.

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DISCUSSION

Sinden: Were all the HI experiments done in adult rats?

Snyder: The normal paradigm for doing this is to take a week-old mouse, ligate the common carotid artery, and then expose the animal for three hours to low oxygen tension, keeping the body temperature normal. This is a classic model for imposing a permanent injury that emulates a common cause of extensive, devastating cerebral palsy. (From an experimental point of view, by the way, this model comes as close as a neurobiologist will be able to come to emulating the haematopoietic stem cell biologist's test of ablating the bone marrow and looking for reconstitution.) Then we would selectively look at non-neurogenic areas; areas that, on the basis of clinical and animal data, never appear to 'repair' themselves.

Rosser: Have you put these cells into any adult models of injury?

Snyder: Yes. We are presently putting these cells in adult stroke and in adult SCI. Actually, one of the earliest examples of the response of NSCs to degeneration was observed in the adult in a series of experiments done with Jeff Macklis a number of years ago (Snyder et al 1997). Jeff has an intriguing model where he can selectively induce a subclass of pyramidal neurons in the adult neocortex to degenerate by apoptotic mechanisms. Cytolytic nanospheres are injected into one hemisphere. The subclass of pyramidal neurons in cortical layers 2 and 5 that send axons across the corpus callosum take up these nanospheres at their target regions and retrogradely transport them back to their cell bodies. If those cells are exposed to a laser beam of a prescribed wavelength, they will die an apoptotic death, leaving the rest of the cortex intact. If we implant an NSC clone into this 'depyramidized' area, a significant proportion will now become pyramidal neurons, although cortical neurogenesis has normally ceased. A subpopulation of these stem cell-derived pyramidal neurons will send their axons back across the corpus callosum to the

appropriate target. Outside of that circumscribed area of neuronal loss, NSCs will *not* yield neurons at all; they will produce the 'normal' post-fetal developmental profile of cells; glial or undifferentiated cells. In other words, the exogenous NSCs seem to mirror faithfully the normal developmental processes prevalent at a given time in a given region; where neurogenesis is ongoing, NSC-derived neurons will be found; where neurogenesis has ceased and gliogenesis predominates, NSC-derived glia will be found. However, we find that if the same terrain is injured, a different scenario unfolds. In the mouse there seems to be a 'window' of one week following injury during which there seems to be a 'resetting' of the 'clock' transiently back to an embryonic environment; then the window closes again.

Gray: In stating that this was apoptotic cell death, is that simply the model you use, or are you implying that signals from apoptotic cell death are critical, and that from necrosis you would get a different set of signals?

Snyder: That is a great question. When Jeff and I first did that experiment, because the results were somewhat unexpected, we thought that, perhaps, it was something particular to apoptosis. It was because of those data that I subsequently approached the spinal cord to investigate the apoptotic degeneration of another selected neural cell type outside its normal period of neurogenesis — the α MN. As we started employing other injury models such as infarcts, I was a little less certain about the singular role of apoptosis. Although we are learning that 'paraptosis' or 'apoptosis' play a prominent role in many degenerative processes, including those that have classically been thought of as predominantly 'necrotic', such as an infarct, and although I can't rule out that it is those apoptotic components that are causing the effects we see, I am beginning to suspect that the altered responses of NSCs to degeneration and injury reflect a broader phenomenon.

Reier: In the MN replacement study, am I right in thinking that you did neurectomies and grafting of the cells at the same time, in the neonate?

Snyder: It is important to recognize that MNs are not normally born beyond fetal life. The model entails first performing a sciatic nerve transection in the neonatal period, but then allowing the animals to mature by which time the MNs degenerate irreversibly. We started implanting NSCs at 4 weeks. Interestingly, the closer the implantation was performed to the actual active degeneration of the MNs, while they were undergoing apoptosis, the more robust the differentiation 'shift' by NSCs to yield MNs was.

Aebischer: Sciatic nerve axotomy is a rather acute model. There are many chronic models for MN degeneration: have you looked at these?

Snyder: We are starting to do this now. Initially, we wanted to examine a model that had a well-characterized, controllable onset and caused a robust, synchronized neuronal death, ideally by an apoptotic mechanisms in a well-defined region. This

is why we chose sciatic nerve axotomy. We are now starting to approach models that perhaps emulate amyotrophic lateral sclerosis (ALS) better, but where the mechanism may be less clear. In early studies in the SOD transgenic mouse, we have observed preliminary suggestions of a prolonged lifespan and diminished symptoms. These preliminary results, of course, require more detailed analysis.

Lindvall: Is there any functional recovery provided by these cells you inject into the penumbra zone or into the infarcted area?

Snyder: We are starting to look at this now. At this initial stage it is probably better to think of these experiments as cell biology that happened to be performed *in vivo*. Second, anyone who has worked with mice knows that even that kind of dramatic experimental defect in the cortex does not give a dramatic functional phenotype. We have not yet done more subtle cognitive testing.

Lindvall: The cortical deficit can be assessed by neurological tests.

Snyder: I agree, and we are starting to do some of those tests. We, however, want to make sure that we do tests that don't just reflect global behaviour. We want to do functional tests that key in specifically to the cells that we were trying to replace, to see whether our cells are being integrated into the circuitry, as opposed just to turning up the gain on parallel systems, or just providing a cellular source of released trophic factors. We are trying to be selective in the tests we employ.

Perry: The glioma seems to be full of interesting puzzles. The phenomenology is clear, but how do you interpret it from the cell biology? Why would a progenitor cell hitch a ride on tumour cells that go wandering off across the brain? Why are the cells migrating towards a tumour?

Snyder: The simple answer is that I don't know. I can come up with the same kind of hypotheses that any of us would entertain. A number of investigators think that tumours are progenitor cells that have gone awry, yet retain some of the same biological properties. One of these is robust migratory behaviour. It may be that the tumour cells infiltrate normal tissue by the same mechanisms that progenitor cells migrate through normal tissue. It may not be that one is 'hitching a ride' on the other, even though it looks like that; they could instead both be responding to similar cues. A second possibility is that the tumour is secreting factors that draw cells to it. This is suggested by some of our *in vitro* work in which tumour cells are permitted to confront stem cells. A third option is that the tumour is causing tissue damage that, in turn, produces signals that attract the progenitors. The migration of these donor NSCs seems to be highly directed. In an adult cortex without a tumour, the cells will engraft well but remain relatively restricted in their distribution and don't have this robust migration. However, the introduction of pathology appears to prompt directed migration to the affected area.

Gray: Does this offer a means of targeting something to a tumour in a manner that might then destroy the tumour cells?

Snyder: That is our hypothesis. The hope is that this could be an adjunctive therapy for gene therapy or other types of interventions against brain tumours.

Blakemore: You have to be very careful to determine whether you are looking at migration or survival. It may just be that you have produced a survival trackway, and this is the only place the cells can go. If this was the case, you could mistake this for directed migration.

Snyder: That is a good point. The total number of cells doesn't appear to change, so we do not believe we are losing cells. It doesn't say that there is not migration; it raises the question of what is causing the migration.

Gray: It is not even about what the causes are — I think Bill is saying that what appears to be a causal process could just be a selective process.

Snyder: We do not appear to be losing cells, such that the appearance of a given population of cells is merely a selection for survival. The distribution of the same number of cells seems to be altered. The real question, as mentioned before, is identifying the forces driving or directing this migration. For example, in the model of spinal MN degeneration in which stem cells implanted into dorsal horn migrate ventrally, one could remark that this is a long way for a diffusible factor to attract cells. An alternative intriguing explanation might hold that the 'inclination' of NSCs to migrate is not altered but rather as the SC starts developing, barriers to migration are progressively established as a normal part of regional specification. With an injury, especially with the selective loss of a particular cell type, there may be a loss of these barriers, a disinhibition, such that exogenous NSCs that ordinarily would have been excluded from migration to that ventral horn region now have the opportunity to enter it and encounter cues that they had been excluded from seeing that led to α MN differentiation.

Blakemore: This is my point: we must not mistake survival for migration.

Finsen: Do any of your cells differentiate into microglial cells?

Snyder: Microglial cells are derived from the bone marrow. We have no evidence that NSCs, when implanted into the brain *in vivo*, can give rise to haematopoietic lineages.

Price: Yesterday we discussed the transient developmental mechanisms, which are involved in generating cells during embryogenesis, but which are absent later on during the process of regeneration. Your MN model is a beautiful system with which you could start to address some of those issues, particularly with regard to the hypothesis you just posed that there might be some sort of barrier. What I would have thought was more likely, rather than some barrier being removed (for which there is currently no evidence), would be thinking about what is taking place of the floorplate during development. The floorplate is crucial in determining the fate during development of exactly the population of cells that you have killed and then replaced. One could hypothesize that somehow the mechanism the floorplate uses is being reactivated in the absence of the floorplate. Isn't an obvious

experiment to ask whether sonic hedgehog is being turned on again during repair? If so, which cells are taking on the role of producing that factor? You might find that you are able to manipulate expression.

Snyder: That is an excellent speculation and we are looking at that. The netrins or the semaphorins may be appropriate candidates for the 'attractants' in this model, but the recapitualted differentiation of MNs is very 'floorplate-esque', and hence suggestive of a role for sonic hedgehog or a related factor.

Rosser: Have you seen any differences between the oncogenically transformed and non-transformed stem cells.

Snyder: Those cells are not transformed, and the term 'oncogenesis' would be totally misused in such a context. For me, as for oncobiologists, 'transformation' has a specific rigorous definition, which is synonymous with the loss of growth control mechanisms (by meeting such established criteria as loss of contact inhibition, ability to grow in soft agar, ability to give rise to tumours in the nude mouse, the inability to respond to normal signals to withdraw from the cell cycle). In stem cell clones in which propagation is assisted by a cell cycle regulatory gene, as soon as the cells enter the brain, that gene product constitutively and spontaneously disappears. To help answer your question, we now have various clones of human NSCs that are either propagated solely with exogenous mitogens (e.g. fibroblast growth factor [FGF] 2) or are propagated by mitogens augmented by the non-transforming propagating gene v-*myc*. V-*myc* operates downstream of and within the same signal transduction pathway as FGF2. It appears to be regulated in the same manner as endogenous cellular *myc* by normal developmental mechanisms including being down-regulated by the cell as it becomes quiescent during mitotic arrest and/or differentiation. Though the cells contain v-*myc*, they are nevertheless dependent on FGF2 for their propagation; in the absence of FGF2, the cells exit the cell cycle and differentiate. We suspect that the function of *myc* may be to prevent the cells from senescing or losing their multipotent phenotype after multiple passages. We are trying to compare these variously propagated clones 'head to head' in all of these paradigms. In every aspect that we have examined so far, the human NSCs with v-*myc* and those without seem to behave identically, even after multiple passages. It frankly remains a matter of speculation in the stem cell field the best way to identify, expand and employ human NSCs. In the absence of reliable, unambiguous, universally agreed upon and widely verified cell surface or other markers, the field still relies upon operational definitions. As indicated in my paper, in collaboration with Richard Mulligan and Lou Kunkel at my institution, we are devising prospective FACS-based methods for isolating NSCs from neural tissue (both rodent and human) that appear to be based on cell cycle properties common to stem cells from all organ systems. (Indeed the established and proven murine and human NSC clones described in the experiments I have referred to in my

presentation segregate to and virtually engorge the 'bin' that selects stem cell populations, and cells in this bin have been shown to be NSCs by criteria employed by even other investigators, e.g. 'sphere formation', etc.)

The loss of v-*myc* expression spontaneously and constitutively from stably engrafted NSCs following transplantation is consistent with the invariant absence of brain tumours derived from implanted v-*myc*-propagated NSCs, even after several years *in vivo* in mice. With human NSCs, as with mouse NSCs, expanded in this manner, neoplasms are never seen. We have begun to entertain the hypothesis that v-*myc* may help to maintain cells in the cell cycle — hence holding their differentiation in abeyance — just long enough for them to exit the cell cycle within the transplanted brain as opposed to the culture dish and hence engraft and integrate optimally having been influenced maximally by their environment. (Cells that exit the cell cycle and pre-differentiate in the dish rather than in the recipient brain tend to engraft quite poorly.) We are also beginning to hypothesize, based on our observations, that v-*myc* may both preclude senescence of NSCs even after multiple passages as well as insuring that the cells maintain their NSC character — i.e. preventing phenotypic 'drift' — from passage-to-passage over prolonged periods of time. If you put v-*myc*-containing mouse cells or human cells into serum-free medium without a defined mitogen, they will come out of the cell cycle and differentiate, so v-*myc* alone is not sufficient to maintain cells in the cell cycle.

Price: The issue surely would be whether the cells are more susceptible to becoming transformed. This is slightly different, and would be the crucial issue if one is thinking in terms of therapy. Transformation is thought to be a multistep process, and you have pushed your cells at least one step closer to transformation. Therefore the probability that a subpopulation of the cells can emerge that were transformed is raised.

Snyder: I actually don't believe that such cells have been pushed a step closer to 'transformation'. As you have indicated, we now appreciate that the development of a neoplasm is a much more complex, multifactorial process than was originally believed in the 1980s. It entails a number of aberrations, beyond just the presence of one or even multiple genes. There are fundamental cellular processes that must go awry. (Indeed, even the term 'oncogene' has become passé — at least among oncobiologists; indeed most of the 'oncogenes' of the 1980s — *bcl-2*, *trk*, *wnt*, *erbB4*, *myc* — have lost that designation as a better understanding of their fundamental cellular role has unfolded.) My bias is that, if the correct genes are used to permit maintenance in the cell cycle while *in vitro* but not *in vivo* — genes that are controlled constitutively and in self-regulated manner by the normal cellular processes that routinely chaperone cell cycle regulatory processes — then there is no more risk (and perhaps less risk) than taking a cell and 'bathing' it chronically in a mitogen. Probably our notions of what it takes to really

'transform' a cell are somewhat naïve. Over 14 years, both in our lab and in the labs of multiple collaborators throughout the world, there have been thousands and thousands of mice transplanted with genetically propagated cells without a single neoplasm, actually a better track record than some growth factor-perpetuated cells after prolonged passages.

It probably bears discussing that, although the term 'transformation' is casually and usually incorrectly tossed about by non-oncobiologists in the NSC field, even manipulated NSC clones never meet the established, rigorous criteria that properly define that term *in vitro* or *in vivo;* such clones respond to and respect all appropriate growth control signals for cell cycle withdrawal, differentiation and interaction with host cells. In culture, they become contact-inhibited, cannot grow in soft agar, contain normal arrestable actin stress fibres and have a normal cell cycle length. When tested in nude mice, neoplasms never develop. In normal grafting studies, brain tumours are never seen; donor-derived cells insinuate themselves seamlessly and non-disruptively into the host cytoarchitecture. They never form inappropriate cell types. Furthermore, the total number of cells (host-plus-donor) observed in a given engrafted region always equals that observed in an analogous region of an untransplanted animal (i.e. host cells alone), suggesting that donor NSCs do not abnormally augment or deform their region of integration, but rather compete equally for space with host progenitors. These observations are in agreement with the finding that, when recipient animals are 'pulsed' with BrdU, the proportion of donor cells that are still mitotic falls to zero by 48–72 h post-engraftment in non-lesioned, non-neurogenic regions, a phenomenon that mirrors their behaviour in culture following contact inhibition. Accordingly, transplanted mice never exhibit neurological dysfunction and CNS regions within which donor cells engraft develop normally. In fact, recent studies have shown that they function in concert with host cells in a physiologically appropriate manner. Indeed, some of the clones of murine NSCs we have used (which happen to be genetically propagated) have been most useful in helping to delineate fundamental stem cell properties (in addition to being among the safest and most efficacious to date).

It actually still remains uncertain the best way to expand and propagate stem cells. There appears to be little doubt that expansion by some technique of the relatively small NSC population that exists in the brain will be required at some stage in the process in order to make therapeutic interventions practical. Our data, especially with human NSCs, that clones propagated by one technique or the other seem to behave virtually identically, suggests that the door has been thrown open for investigators and/or clinicians to pick the technique that best serves their clinical or research demands. Importantly, these findings have helped unify various research directions in this field: insights from studies of NSCs perpetuated by one technique can now be legitimately joined to those derived

from studies employing others, providing a more complete picture of NSC biology and its applications. (In fact, for some needs, because the key to exuberant engraftment is insuring that NSCs exit the cell cycle at precisely the right time — within the parenchyma, not outside it — self-regulated genetic means may actually prove to be the easier, safer, more cost-effective and reliable of strategies. Furthermore Svendsen has begun to marshal evidence that mitogen-mediated expansion alone may confront a Hayflick-like phenomenon of inherent senescence that may require blunting by genetic means.)

Whether re-deriving stable, well-characterized clones for each clinical situation is prudent or effective by whatever means remains to be empirically determined. While the field has been contemplating of late the value and feasibility of isolating adult NSCs from a given prospective recipient for subsequent autologous grafting, it should be noted that, for neurodegenerative diseases of possible genetic aetiology or predisposition — among them, Parkinson's disease — one clearly would *not* employ such a strategy. Indeed, the most effective and safest NSC clones (human or otherwise) — and the tacit goal within the stem cell community — is to derive human NSCs that can serve as 'off-the-shelf' reagents and behave like established, stable, physiologically normal, well-characterized, homogeneous, readily accessible, abundant and *universally tolerated* cell lines. Regardless of how these debates settle out over the next few years of empirical study, the human NSC clones described by us here — if not the cells that actually go to clinical trials — can clearly serve as prototypes and the experiments in which they are used as proofs-of-principle for reporting on the efficacy of NSCs in these neurodegenerative environments. Based on our results to date using such murine and human NSCs in various disease models, they certainly can help establish a therapeutic standard that should be at least met by any method proffered for generating NSCs for clinical use. To achieve less may mean failing to truly realize or unlock the capabilities of the NSC.

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Isolation and Intracerebral Grafting of Nontransformed Multipotential Embryonic Human CNS Stem Cells

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ABSTRACT

In this work, we show that the embryonic human brain contains multipotent central nervous system (CNS) stem cells, which may provide a continuous, standardized source of human neurons that could virtually eliminate the use of primary human fetal brain tissue for intracerebral transplantation. Multipotential stem cells can be isolated from the developing human CNS in a reproducible fashion and can be exponentially expanded for longer than 2 years. This allows for the establishment of continuous, nontransformed neural cell lines, which can be frozen and banked. By clonal analysis, reverse transcription polymerase chain reaction, and electrophysiological assay, we found that over such long-term culturing these cells retain both multipotentiality and an unchanged capacity for the generation of neuronal cells, and that they can be induced to differentiate into catechlaminergic neurons. Finally, when transplanted into the brain of adult rodents immunosuppressed by cyclosporin A, human CNS stem cells migrate away from the site of injection and differentiate into neurons and astrocytes. No tumor formation was ever observed. Aside from depending on scarce human neural fetal tissue, the use of human embryonic CNS stem cells for clinical neural transplantation should provide a reliable solution to some of the major problems that pertain to this field, and should allow determination of the safety characteristics of the donor cells in terms of tumorigenicity, viability, sterility, and antigenic compatibility far in advance of the scheduled day of surgery.

Key words: cell replacement, human stem cells, neural transplantation, EGF, FGF2

INTRODUCTION

ANY EXPERIMENTAL REPAIR STRATEGY aimed at the development of therapies for the cure of neurodegenerative disorders must take into account the inherent lack of regenerative capacity of the central nervous system (CNS) of adult mammals. Neural transplantation represents one of the most innovative approaches directed at restoring neurological function through the replacement of cells lost to injury or disease by means of intracerebral grafting of embryonic cells, which possess the necessary differentiation potential and plasticity to functionally integrate into the damaged neural circuitry (Brüstle and McKay, 1996; Gage and Christen, 1997). In this perspective, implantation of embryonic CNS precursors is currently under active investigation in various animal models of neurological disorders, including metabolic deficit (Snyder et al., 1995), Huntington's disease (Peschanski et al., 1995), and Parksinson's disease (Olanow et al., 1996) as well as spinal cord injuries (Anderson et al., 1995; Mori et al., 1997).

The development of intracerebral transplantation in hu-

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mans is presently hampered by the need of using human brain tissue of fetal origin. The scarcity of this material is compounded by practical issues such as age of the donor, viability, contamination, and heterogeneity of tissue as well as overwhelming ethical and moral concerns (Björklund, 1993).

It has been proposed that neural precursor expanded in culture may represent a suitable alternative to fetal tissue in brain transplantation. In this perspective, CNS stem cells (Davis and Temple, 1994) appear to be an elective choice because of their extended proliferative potential and their capacity to generate all three main brain cell types (Reynolds and Weiss, 1992; Gritti et al., 1996).

In this work, we present data concerning the isolation, characterization, and manipulation of multipotential stem cells from the human embryonic CNS and show that they can effectively be expanded in an undifferentiated state for over 2 years *in vitro*. During this time, they retain stable functional features, which means that they retain both a steady growth profile and multipotentiality. Human CNS stem cells are plastic and can be induced to differentiate into neurons displaying a catecholaminergic phenotype. Following transplantation into the adult rat brain, these cells survive and differentiate into neurons and astrocytes.

METHODS AND RESULTS

Since the basic conditions that allow for the growth of rodent stem cells were not sufficient for the culturing of human CNS stem cells (Svendsen et al., 1996; Chalmers-Redman, 1997), specific culture conditions had to be established. Diencephalic and cortical stem cells were isolated from 10.5-week post conception human embryos, by mechanical dissociation, and could be cultured in NS-A basal medium (Euroclone, Scotland) containing 2 mM l-glutamine, 0.6% glucose, 9.6 μg/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml transferrin, and 2 μg/ml of heparin (sodium salt, grade II, Sigma) in the presence of both epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2), at a final concentration of 20 ng/ml and 10 ng/ml, respectively. The required simultaneous exposure to both growth factors (GFs) is an identifying feature that distinguishes human stem cells from their rodent counterparts, as the latter can be cultured in the presence of each of these GFs alone (Reynolds and Weiss, 1992; Gritti et al., 1996).

Two to 4 days after plating at less than 5×10^4 cells/cm^2, cell death rapidly ensued. However, a minor subset of the total cell plated entered an active mitotic state, proliferated, and gave rise to spherical, floating clones of cells called neurospheres, which first appeared between 7 and 15 days. Cells within the spheres were negative for markers of neural differentiation but expressed the neural precursor antigen nestin. Cultures could then be harvested, mechanically dissociated, and replated as a single-cell suspension under the same culture conditions. New spheres were generated by 8–12 days, depending on the area of origin of the cells. Human stem cell cultures could be serially passaged in this manner for over 2 years, yielding an exponential, consistent increase in the total cell number. Over this time, human CNS stem cells retained nestin expression and lacked specific markers of differentiation. However, by clonal analysis, these cells were shown to be multipotential. A single cell monitored by time-lapse microphotography was shown to generate a neurosphere (Fig. 1), whose progeny were plated onto an adhesive substrate and allowed to spontaneously differentiate following removal of GFs. By indirect immunocytochemistry, a single stem cell was seen to give rise to neurons, astrocytes, and oligodendrocytes, thus showing its multipotentiality. The fact that similar results were obtained when clonal analysis was repeated at increasing subculture passages

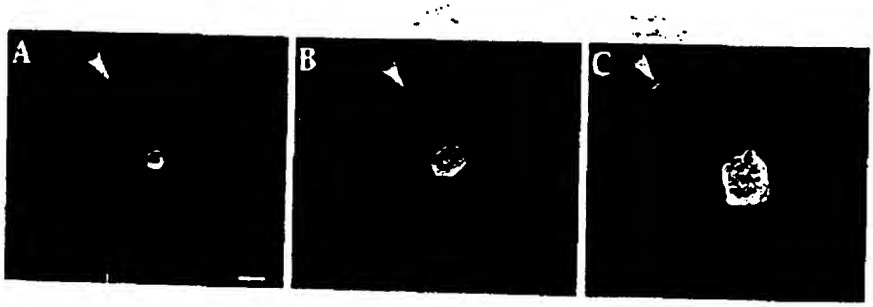

FIG. 1. A single stem cell from the human presumptive diencephalon (10.5 weeks post conception) is shown 1 day after plating onto polyornithine-coated dishes in growth medium (A; the landmark identifies the field). THe cell proliferated (B; 9 DIV) and by 23 DIV, gave rise to a spherical clone of cells (C) that could then be subcultured or differentiated into neurons and glia. Bars = A–C 20 μm, bar in A.

demonstrates that multipotentiality is an intrinsic feature of human stem cells that is stably retained over time.

Self-renewal is an essential feature of stem cells (Loeffler and Potten, 1997). Self-renewal can be achieved by fixed asymmetric divisions in which one stem cell gives rise to one differentiated cell and to one offspring identical to itself, or at the level of cell population by a mix of asymmetric divisions and symmetric cycles in which either two differentiated daughters or two stem cells are produced. In a population that expands in culture, multiple symmetric division of the last kind must occur. We showed this by a clonogenic assay, in which a clonal sphere (Fig. 1) was dissociated and its progeny replated at clonal density to give rise to secondary spheres. These were then either subcloned once more, or differentiated and immunolabeled for neuronal and glial antigens. These experiments showed that a single human neural stem cell generates multiple secondary stem cells that are identical to itself and that preserve multipotential features. Hence, human stem cells are capable of self-renewal and achieve this function by undergoing multiple symmetric divisions in which two stem cells are generated.

Quantitative immunofluorescence analysis of differentiated cell subtypes 4 days after differentiation was initiated showed that 13%, 75%, and 1.2% of the stem cell progeny differentiated into neurons (by anti-β-tubulin-immunoreactivity; -IR), astrocytes (by glial fibrillary acidic protein-IR; GFAP), and oligodendrocytes (galactocerebroside-IR; GC), respectively. Neuronal differentiation was confirmed by the immunochemical and molecular demonstration of additional lineage markers such as microtubule-associated proteins 2 and 5, Tau-1 protein, neuron-specific enolase and neurofilaments, and by the detection of cells endowed with typical neuronal electrophysiological features, including the capacity to elicit bona fide action potentials that were reversibly inhibited by tetradotoxin and 4AP. More importantly, the number of neurons generated by serially subcultured human CNS stem cells did not change over time, as the same percentage of neuronal cells could be identified in human CNS stem cell cultures at early and late passages (13% and 12.7%, respectively). Altogether, these findings demonstrate that stem cells can be isolated from the embryonic human CNS and can be consistently expanded in an undifferentiated state by means of GF-mediated epigenetic stimulation. Over long-term subculturing, these stem cells retain multipotentiality, consistent growth features, and an unchanged spontaneous capacity for neuronal differentiation. Additionally, human CNS stem cells can be cryopreserved easily and efficiently for extended period of times.

When differentiated stem cell progeny were analyzed for expression of specific neurotransmitter characteristics, only the GABA-ergic phenotype could be confirmed by both detection of glutamic acid decarboxylase (67kD isoform) and GABA. However, the differentiation potential of human CNS stem cells from both diencephalon and cortex appeared to be broader than expected. In fact, both stem cell types could be induced to express the catecholamine synthesis–limiting enzyme tyrosine hydroxylase (TH). Expression of TH occurred in cells expressing the neuronal marker β-tubulin, however, was not a spontaneous process, as it required the simultaneous exposure of cells undergoing differentiation to both FGF2 and Sato/N2 serum-free medium conditioned by a 80% confluent rat glioma cell line (BB49) for 48 h. TH induction was gradual, peaked at a value of 6% of the total cell number by 5–7 days following induction, and occurred in approximately 30% of the differentiated neuronal progeny (Vescovi et al., 1997). Expression of TH was confirmed at the RNA and protein level by reverse transcription polymerase chain reaction, immunocytochemistry, and Western blotting (Fig. 2). Removal of FGF2 and glial conditioned medium did not result in loss of TH expression. Thus, the plasticity of human CNS stem cells allows for specific neuronal subtypes to be generated under specific differentiation conditions.

The induction of the catecholaminergic phenotype in stem cells derived from human neurons underlines how human stem cells may be used for intracerebral transplantation in Parkinson's disease as well as other neurological disorders. Hence, in a series of preliminary experiments, we attempted to demonstrate the engraftability of long-term cultured human CNS stem cells. Passages 26 through 34 human diencephalic stem cell cultures were harvested 4–6 days after the induction of differentiation by GF removal and plating onto polyornithine-coated plastic dishes. Cells were labeled while dividing in vitro for 6 days in the continuous presence of 0.75 μM 5-bromodeoxyuridine (BrdU) and were mildly dissociated by extrusion through a narrowed, fire-polished glass pipette. Then, 1–3 μl of a 2.5×10^7 cell/ml suspension were transplanted into the ipsilateral striatum of adult Sprague-Dawley rats via a 30 gauge cannula at a controlled rate of 0.1–0.5 μl/min. Stereotaxic coordinates were A/P, +0.5; M/L, −3.1; and D/V, −4.8. Animals were chronically immunosuppressed by administration of cyclosporin cyclosporinA (Sandimmune; 0.1 mg/ml) in their drinking water. Detection of injected cells was performed by immunostochemistry with an anti-BrdU antibody. Four and a half months following transplantation, 12.3% of the total cell injected could be detected by anti-BrdU immunolabeling, which had migrated as far as 1.2 mm rostrally and 0.9 mm radially from the injection site. Double immunocytochemistry showed the

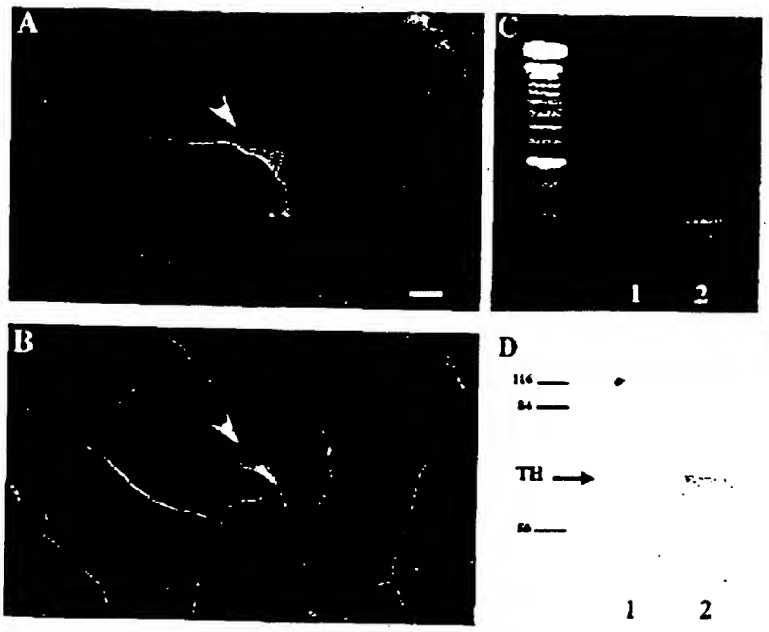

FIG. 2. Serially subcultured human diencephalic stem cells (passages 20 through 36) were differentiated either by growth factor removal in serum free medium (control) or in the presence of medium conditioned by B49 glioma cells supplemented with 10 ng/ml of FGF2 (TH-induced). Double labeling immunofluorescence demonstrated the presence of the tyrosine hydroxylase (TH) protein (A) in neuronal cells immunoreactive for the early neuronal marker β-tubulin (B). The expression of TH was confirmed to occur in TH-induced cells at both the mRNA (C, lane 2) and the protein level (D, lane 2), but not in control cultures (C, lane 1; D, lane 1). Bars = A, B 20 μm, bar in A.

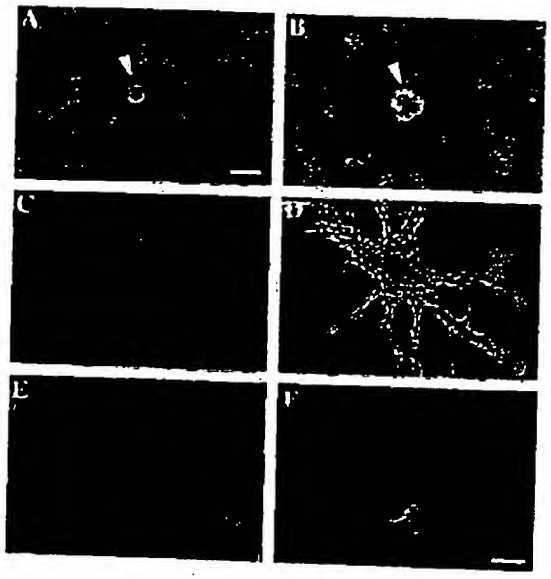

FIG. 3. Stem cells could be isolated from the subventricular region of the lateral ventricles of adult nonhuman primates (*Macaca mulatta*). Single hypertrofic cells (A) proliferated and gave rise to clusters of cells (B, C) that, following serial subculturing, were plated onto polyornithine-coated glass (D) coverslips and differentiated to generate astroglial (E; GFAP) and neuronal cells (F; MAP2). Bars = A–D 20 μm, barin A; E, F 20 μm, bar in F.

presence of cells expressing either the neuronal marker NSE or the glial antigen GFAP among the BrdU-labeled cells. Neuronal differentiation was further confirmed by the detection of cells expressing the human specific neurofilament antigen. Tumor formation was not observed.

DISCUSSION

These data confirm that human stem cell progeny can survive transplantation into the adult rodent brain, migrate from the transplantation site, and differentiate into astrocytes and neurons. These findings support their systematic use as donor tissue for neural transplantation, as they may offer several advantages to preclinical and clinical studies. Human CNS stem cells provide control over variables that currently affect clinical neural transplantation, such as cell viability and the composition of donor material. Additionally, since differentiation can be initiated by varying the culture conditions, donor cells can be harvested and transplanted at specific stages of maturation. Biosafety and histocompatibility features can be predetermined far ahead of the day of surgery (Olanow et al., 1996).

Although the use of human CNS stem cells will significantly reduce the need for fetal tissue, a definitive answer to the moral and ethical issues raised by the use of this material may come only from the use of stem cells derived from the human adult CNS as they may be used for therapeutic autologous transplantation. In a set of preliminary experiments, we applied the above methodology to cultures established from the subventricular zone of the lateral ventricles of adult nonhuman primates (*Macaca mulatta*). Intriguingly, cells that underwent proliferation when exposed to EGF and EGF2 could consistently be isolated from this region. However, cell proliferation was extremely slow, and the doubling time for these cultures exceeded 15 days *in vitro*. Similarly to their rodent and human embryonic counterparts, monkey SVZ cells ceased proliferation and rapidly differentiated when GFs were removed from the culture medium. Both astroglial and neuronal cells could be detected within the differentiated progeny. Unfortunately, the vast majority of the cells appeared to differentiate into astroglial cells as demonstrated by expression of GFAP, while the detection of cells expressing the neuronal markers MAP2 and Tau-1 was infrequent (less than 1% of total cell number; Fig. 3). Hence, although it appears that the adult primate brain contains neural precursors capable of neuronal and glial differentiation, it is clear that the proper conditions for efficient isolation, expansion, and differentiation of these cells still have to be identified and require further investigation. Nevertheless, as our understanding of their basic physiology improves, new therapeutic approaches to neurological disorders will be developed in which the use and manipulation of stem cells may play a pivotal role.

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